UNIVERSIDAD AUTÓNOMA DE MADRID

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TESIS DOCTORAL

"PROTECTIVE FUNCTION OF SPECIFIC MICRONUTRIENTS AGAINST CARDIOVASCULAR AND NEURODEGENERATIVE DISEASES"

IMDEA Alimentación

(Instituto Madrileño de Estudios Avanzados en Alimentación)

MARÍA DEL CARMEN CRESPO LORENZO

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(IMDEA Food)

Memoria presentada por

Mª Del Carmen Crespo Lorenzo

Para optar al grado de:

DOCTOR EN FARMACOLOGÍA Y FISIOLOGÍA

Directores de la Tesis:

Dr. Francesco Visioli y Dr. Alberto Dávalos

Investigadores Senior del Instituto IMDEA Alimentación

D. FRANCESCO VISIOLI, DR. EN BIOTECNOLOGÍA POR LA UNIVERSIDAD DE BRESCIA (ITALIA) Y D. ALBERTO DÁVALOS, DR. EN FARMACIA POR LA UNIVERSIDAD COMPLUTENSE DE MADRID, ACTUALMENTE INVESTIGADORES DE IMDEA ALIMENTACIÓN

INFORMAN:

Que el presente trabajo titulado "Protective function of specific micronutrients against cardiovascular and neurodegenerative diseases" que constituye la memoria que presenta Dª. María Del Carmen Crespo Lorenzo para optar al grado de Doctor en Farmacología y Fisiología, ha sido realizada en el Instituto Madrileño de Estudios Avanzados en Alimentación (IMDEA Alimentación) bajo su dirección, y cumple las condiciones exigidas para optar al grado de Doctor por la Facultad de Medicina de la Universidad Autónoma de Madrid.

Y para que así conste, firman el presente informe:

Dr. Francesco Visioli

Dr. Alberto Dávalos

This work was developed in the framework of two Spanish State Program projects whose references are:

- AGL2011-28995 titled “Foodomics of hydroxytyrosol: insights into its molecular effects and search of new markers and targets” at Instituto Madrileño De Estudios Avanzados en Alimentación (IMDEA Food).
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Don`t let your happiness depend

on something you may lose.

Cs. Lewis

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Publication II: **Crespo MC**, Tomé-Carneiro J, Burgos-Ramos E, Loria Kohen V, Espinosa MI, Herranz J, Visioli F. One-week administration of hydroxytyrosol to humans does not activate Phase II enzymes. Pharmacological Research 95-96:132-7 (2015).

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ABSTRACT

Aging is one of the most important risk factors for cardiovascular (CVD) and neurodegenerative diseases (ND), which are the main cause of hospitalization, permanent disability, and death for Europeans over 65 years. These diseases are due to various causes such as physical inactivity, obesity, diabetes, hypertension and unbalanced diets. Several epidemiological studies clearly show that proper diets are associated with lower incidence of cardiometabolic disorders, cancer, and ND. Therefore, maintaining adequate nutrition or incorporating nutritional supplements could be important to prevent CVD and the early onset of ND. Some diets are healthier, e.g. the Mediterranean and Japanese ones, than others and certain typical micronutrients consumed in these regimens were studied here, namely soy isoflavones (SI), hydroxytyrosol (HT), and polar lipids (PLs) of marine and dairy origins enriched in phospholipids (PHLs). The ultimate goal was to find solid scientific, i.e. molecular, genomic, and proteomic, as well as human evidence to back up dietary advice and the formulation of nutraceuticals and functional foods.

Soy consumption has been suggested to afford protection against CVD. We investigated the nutrigenomic actions of **SI**, namely a genistein/daidzein mix (in nutritionally relevant amounts) in young mice with a specific focus on the adipose tissue, due to its pivotal role in cardiometabolism. These compounds have contradictory nutrigenomic effects and differentially expressed genes have diverse effects in these tissues. Moreover, we report increased leptin and total cholesterol, and decreased triglyceride (TG) plasma concentrations.

There are several studies demonstrating that **HT** has numerous potentially beneficial effects in the cardiovascular system, in addition to antimicrobial, anticancer, anti-inflammatory, and neuroprotective properties. In our human studies, twenty-one volunteers were randomly assigned to one of three groups: placebo, 5 or 25 mg of HT per day. We were unable to record significant effects on a variety of surrogate markers of CVD and oxidation. We wanted to analyze if the body processes these polyphenols as xenobiotics via the Keap1/Nrf2/ARE signaling axis (which is currently the most prevalent theory to explain the benefits attributed to polyphenols), leading to the induction of Phase II enzymes. We saw that gene expression of Phase II enzymes is not significantly modified in peripheral blood mononuclear cells. We also evaluated HT bioavailability following ingestion and found that it is bioavailable and is recovered in the urine chiefly as sulphate-3′. In young mice studies supplemented with nutritionally relevant amounts of HT, for eight weeks, we investigated CVD markers and ran a proteomic analysis, in addition to studying whether HT could modulate the expression of microRNAs (miRNAs) in the intestine. We reported that HT increased TG levels. Numerous miRNAs were modulated in the intestine and the analysis of other tissues revealed consistent HT-induced modulation of only few miRNAs. Acute treatment of mice with HT and

experiments in different intestinal cells lines provided mechanistic insights of this induction. HT-induced expression of one miRNAs was also confirmed in healthy volunteers supplemented with this compound. Finally, proteomic analysis of metabolically active tissues (adipose and liver), using the super-SILAC method, showed that some oxidative stress-related proteins were modulated in both tissues. Peroxiredoxin 1 is among the main ones, presenting a consistent repression and, in some cases, a tissue-dependent modulation. To research the effect of this compound on ND, we hypothesized that HT could exert beneficial effects on insulin resistance (IR) associated with Alzheimer's disease (AD). An astrocytic cell line was exposed to amyloid beta peptide (Aβ) (25-35) and co-incubated with HT for different periods. Viability was significantly decreased by Aβ, but both pre- and post-treatment with HT prevented this effect and improved insulin sensitivity, restoring insulin-signaling.

Low consumption of omega 3 fatty acids and PHLs is associated with altered cognitive function at aging. Dietary supplementation with bioactive **PLs** concentrates of marine (KOC) and dairy (BMFC) origins could be an effective strategy for preventing ND. During three months, aged Wistar rats were supplemented with KOC, BMFC, or the combination of both. We analyzed mitochondrial activity and insulin and synaptic signaling in brain. Dietary supplementation with KOC and/or BMFC improves peripheral and central IR and increases brain-derived neutrophic factor (BDNF) levels, favoring an improvement in the energy state within neurons and facilitating both mitochondrial and protein synthesis, which are necessary for synaptic plasticity. Finally, we wanted to see whether PLs could influence the expression of genes or miRNAs related to synaptic activity and/or neurodegeneration. BMFC differentially modulated the expression of 23 miRNAs, and both supplements combined modulated the hippocampal expression of 119 miRNAs. 38, 58, and 72 genes were found to be differentially expressed in the groups supplemented with KOC, BMFC and BMFC + KOC, respectively. Functional analysis of differentially expressed genes showed these were mainly involved in neuroactive processes in the KOC and BMFC groups, and associated with lysosomes and mRNA surveillance pathways in the BMFC + KOC group. Thus, provision of certain PLs could favor neuronal health and delay cognitive decline associated to age.

In conclusion, the results of this doctoral work add to the body of evidence that shows how the intake of certain micronutrients can lessen the burden of degenerative diseases (either vascular/cardiac or cerebral) and suggest the potential use of supplements in addition to proper lifestyle, diet, and medicine.

RESUMEN

El envejecimiento es uno de los factores de riesgo más importantes para las enfermedades cardiovasculares (CVD) y neurodegenerativas (ND), las cuales son, en conjunto, la principal causa de hospitalización, discapacidad y muerte para los europeos mayores de 65 años. Las causas de estas enfermedades se deben a varios factores como, por ejemplo, inactividad física, obesidad, diabetes, hipertensión y dietas desequilibradas. Varios estudios epidemiológicos muestran que seguir una adecuada alimentación está asociado con una menor incidencia de trastornos cardiometabólicos, cáncer y ND. Por lo tanto, mantener una nutrición adecuada o incorporar suplementos nutricionales puede ser importante para prevenir CVD y la aparición temprana de ND. Las dietas mediterránea y japonesa se describen como saludables. Por estas razones, nos planteamos estudiar ciertos micronutrientes presentes en estas dietas: las isoflavonas de soja (SI), el hidroxitirosol (HT) y los lípidos polares (PLs) de orígenes lácteo y marino, enriquecidos en fosfolípidos (PHLs), para investigar sus posibles efectos beneficiosos frente a estas enfermedades.

Se ha sugerido que el consumo de soja proporciona protección contra las CVD. En este estudio se investigaron las acciones nutrigenómicas de las **SI**, genisteína/daidzeína (en cantidades nutricionalmente relevantes) en ratones jóvenes, con un enfoque específico en el tejido adiposo debido a su papel en el cardiometabolismo. Este compuesto presentó efectos nutrigenomicos contradictorios, con una expresión diferencial de genes asociados con efectos diversos en estos tejidos. Además, se observó un aumento de colesterol total y de leptina y una disminución en los niveles de triglicéridos (TG) plasmáticos.

Existen numerosos estudios que han demostrado que el **HT** posee numerosos efectos potencialmente beneficiosos sobre el sistema cardiovascular, además de propiedades antimicrobianas, anticancerígenas, antiinflamatorias y neuroprotectoras. En un estudio realizado durante esta tesis, se reclutaron 21 voluntarios, que se asignaron aleatoriamente al grupo placebo, 5 ó 25 mg de HT por día. No se registraron efectos estadísticamente significativos en una variedad de marcadores de CVD y oxidación. Analizamos si el HT es procesado por el cuerpo como xenobiótico a través de la inducción de enzimas de Fase II en las células mononucleares de sangre periférica, pero no se encontraron diferencias significativas. Se evaluó también la biodisponibilidad del HT después de la ingestión, comprobándose que este es biodisponible y se recupera en la orina principalmente como sulfato-3'. En estudios con ratones suplementados con HT, durante 8 semanas, se analizaron marcadores de CVD, se realizó un análisis proteómico y se investigó si el HT podría modular la expresión de los microARNs (miRNAs). Se observó un aumento en los niveles de TG en plasma. También se vio una modulación de numerosos miRNAs en el intestino, así como en otros tejidos. La expresión inducida de uno de estos miRNAs se confirmó también en el estudio en humanos. El tratamiento agudo con HT en ratones, y experimentos en diferentes líneas celulares

intestinales, proporcionaron conocimientos mecánicos acerca de esta inducción. Finalmente, el análisis proteómico en tejidos metabólicamente activos (tejido adiposo e hígado), mediante la técnica super-SILAC, reveló que algunas proteínas relacionadas con el estrés oxidativo fueron moduladas en ambos tejidos (ej. peroxiredoxina 1, cuya expresión se encontró consistentemente reprimida) y en algunos casos la modulación fue dependiente de tejido (ej. proteínas de síntesis de ácidos grasos). Para investigar el efecto de este compuesto en ND, planteamos la hipótesis de que el HT podría ejercer efectos beneficiosos sobre la resistencia a la insulina (IR) asociada a la enfermedad de Alzheimer (AD). Una línea celular astrocítica se expuso al péptido beta amiloide (Aβ) (25-35) y se co-incubó con HT durante diferentes tiempos. La viabilidad se redujo con Aβ, pero tanto el pre- como el pos-tratamiento con HT evitaron este efecto y mejoraron la sensibilidad a la insulina.

El bajo consumo de ácidos grasos omega 3 y PHLs se asocia con la una función cognitiva alterada en el envejecimiento. La suplementación dietética de **PLs** de origen marino (KOC) y lácteo (BMFC) podría ser una estrategia efectiva para prevenir las ND. Durante tres meses, ratas viejas fueron suplementadas con KOC, BMFC o la combinación de ambas. Analizamos la actividad mitocondrial, la señalización de la vía de la insulina y la vía sináptica en el cerebro. La suplementación dietética con KOC y/o BMFC mejora la IR central y periférica y aumenta los niveles del factor neurotrófico derivado del cerebro (BDNF), favoreciendo una mejora en el estado de energía neuronal y facilitando la síntesis mitocondrial y de proteínas, que son necesarias para la plasticidad sináptica. Analizamos, en hipocampo, la expresión de genes o miRNAs relacionados con la actividad sináptica. La suplementación con BMFC moduló la expresión de 23 miRNAs, y la suplementación con ambos concentrados resultó en la modulación de 119 miRNAs. Se encontró que 38, 58 y 72 genes se expresaron diferencialmente en los grupos suplementados con KOC, BMFC y BMFC + KOC, respectivamente. Según el análisis funcional los genes expresados diferencialmente en los grupos KOC y BMFC participan en procesos neuroactivos y aquellos encontrados en el grupo BMFC + KOC se asocian con lisosomas. Por lo tanto, estas suplementaciones dietéticas podrían favorecer la salud neuronal y retrasar el deterioro cognitivo asociado a la edad.

En conclusión, los resultados de éste trabajo doctoral se suman al conjunto de evidencias que avalan efectos positivos frente a las enfermedades degenerativas (ya sean vasculares/cardíacas o cerebrales) debido a la ingesta de ciertos micronutrientes, y sugieren que el consumo de suplementos puede complementar las pautas recomendadas respeto a la adopción de estilos de vida saludables.

LIST OF ABBREVIATIONS

AD: Alzheimer's disease.
ATP: Adenosine triphosphate.
Aβ: Amyloid β-peptide.
BDNF: Brain-derived neurotrophic factor.
BM: Butter milk.
BMFC: Bioactive phospholipids concentrate of BM.
BMI: Body mass index.
BP: Blood pressure.
BW: Body weight.
cDNA: Complementary deoxyribonucleic acid.
CMD: Cardiometabolic disorders.
COX-2: Cyclooxygenase-2.
CSF: Cerebrospinal fluid.
CVD: Cardiovascular diseases.
DBP: Diastolic blood pressure.
DHA: Docosahexaenoic acids.
DIGE: Differential in gel electrophoresis.
DNA: Deoxyribonucleic acid.
dNTPs: Deoxynucleotide triphosphate.
EFSA: European food safety authority.
EPA: Eicosapentaenoic acid.
ETC: Electron transport chain.
FAO: Food agriculture organization.
FAs: Fatty acids.
FDA: Food and drug administration.
GRAS: Generally regarded as safe.
HD: Huntington's disease.
HDL: High-density lipoprotein.
HOMA: Homeostasis model assessment.
Hs-CRP: High sensitivity C-reactive protein.
HT: Hydroxytyrosol.
IR: Insulin resistance.
IRS: Insulin receptor substrate.
KO: Krill oil.
KOC: Bioactive phospholipids concentrate of KO.
LDL: Low-density lipoprotein.
MAPK: Mitogen actived protein kinase.
MCI: Mild cognitive impairment.
MCP1: Monocyte chemoattractant protein 1.
M-CSF: Macrophage colony-stimulating factor.
MeSH: Medical subject heading.
MFGM: Milk fat globule membrane.
miRNAs: MicroRNAs.
mRNA: Messenger RNA.
MS: Mass spectrometry.
mtDNA: Mitochondrial deoxyribonucleic acid.
MUFAs: Monounsaturated fatty acids.
n-3: Omega-3 fatty acids.
ND: Neurodegenerative diseases.
NFT: Neurofibrillary tangle.
NGS: Next generation sequencing.
NOAEL: No observed adverse effect level.
NP: Neuritic plaque.
OE: Oleuropein.
Ox-LDL: Oxidized low-density lipoprotein.
PC: Phosphatidylcholine.

PCR: Polimerase chain reaction.
PD: Parkinson's disease.
PE: Phosphatidylethanolamine.
PHLs: Phospholipids.
PI: Phosphatidylinositol.
PI3K: Phosphoinositol 3 kinase.
PLE: Pressurized liquid extraction.
PLs: Polar lipids.
PS: Phosphatidylserine.
PUFAs: Polyunsaturated fatty acids.
RCTs: Randomized controlled trials.
RISC: RNA induced silencing complex.
RNA: Ribonucleic acid.
ROS: Reactive oxygen species.
RT: Reverse transcription.
SBP: Systolic blood pressure.
SCORE: Systemic coronary risk estimation.
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis.
Seq: Sequencing.
SI: Soy isoflavones.
SILAC: Stable isotope labeling with aminoacids in cell culture.
SLs: Sphingolipids.
SM: Sphingomyelin.
TAU Protein: Microtubule-associated potein TAU.
TG: Triglycerides.
TICE: Trans-intestinal cholesterol excretion.
TNF-α: Tumor necrosis factor-alpha.
TXB2: Thromboxane B-2.
VEGF: Vascular endothelial growth factor.
WC: Waist circumference.
WHO: World Health Organization.
WHR: Waist-to-hip ratio.
WHtR: Waist to height ratio.

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INTRODUCTION

This PhD work focused on the pharma-nutritional effects of selected food components, with *in vitro*, *in vivo* and human investigations, spanning from nutrigenomic actions to molecular outcomes with potential influence in cardiovascular and neurodegenerative diseases. The results of this work add to and expand the body of evidence in support of rational dietary advice.

1. Studied diseases

1.1. Cardiovascular diseases

Cardiovascular diseases (CVD) are a group of disorders that affect both the circulatory system and the heart. Within this group, we find coronary heart disease (heart attacks), cerebrovascular disease, increased blood pressure, peripheral vasculopathy, rheumatic heart disease, congenital heart disease, heart failure and cardiomyopathy. CVD take the lives of 17.7 million people every year, i.e. 31% of all global deaths, primarily manifesting as heart attacks and stroke (1). These numbers will increase to 23.3 million in 2030 and the incidence of CVD is expected to remain the leading cause of death (2).

Cardiovascular risk factors are those associated with a higher probability of suffering from cardiovascular disease. This concept was introduced in the Framingham study (3), more than 50 years ago. Since then, other epidemiological studies have been conducted in populations to identify different risk factors. The most commonly used tables to calculate the overall cardiovascular risk of an individual are those from the Framingham study and the ones derived from the Systemic Coronary Risk Estimation (SCORE) project. These tables are a scoring system which calculates a 10 year-risk of fatal CVD. Their use is recommended for

risk assessment and can help make logical decisions and avoid both excess and lack of treatment.

Risk factors are conveniently divided in two main types: 1) non-modifiable factors, such as genetic inheritance, sex, age, race, and 2) modifiable factors, such as hyperlipidemia, diabetes, hypertension, smoking, alcohol, stress, obesity and heart rate (4, 5).

Many CVD, as angina pectoris, myocardial infarction and peripheral vascular disease are caused by atherosclerosis, which is a chronic inflammatory process that occurs in the wall of the large arteries in response to an "attack" to the endothelium. Atherosclerosis can affect any artery in the body, including those of the heart, brain, arms, legs, pelvis, and kidneys. Atherosclerosis is characterized by the presence of fibro-fatty plaques that begin to develop in the arterial intima layer. The atheromatous plaque mainly develops in aorta, coronary arteries and cerebral arteries, but can invade the arterial lumen and cause blockage of blood flow, making it difficult for blood to irrigate tissues and causing ischemia. Low-density lipoproteins (LDL), the major carriers of cholesterol, are deposited within the atheroma plaques. Elevated fasting glucose and lipids levels, postprandial hyperglycemia and hypertriglyceridemia are considered as cardiovascular risk factors (6). The accumulation of these particles stimulates the endothelium to produce pro-inflammatory molecules, including adhesion molecules (VCAM-1, PCAM-1, ICAM-1, P-Selectin, E-Selectin), chemotactic proteins, for example monocyte chemoattractant protein 1 (MCP-1) and growth factors, macrophage colony-stimulating factor (M-CSF). Together, this process is known as endothelial activation and is mediated largely by the nuclear factor NF-κB (7). In response to these chemotactic factors, immune cells (mainly circulating monocytes) penetrate the

wall of the artery and differentiate into macrophages, which have surface scavenger receptors, such as SR-A and CD-36 that recognize and capture modified lipoproteins (8). Macrophages begin to phagocyte and accumulate lipids and end up becoming foam cells. The uptake of LDL particles by the scavenger receptors requires their prior modification possibly by oxidation due to free oxygen radicals produced in the endothelium or extracellular matrix and by macrophages.

The atheroma plaque may be fragile and rupture, thereby secreting extracellular matrix into the bloodstream and forming a thrombus (embolism), thus occluding the circulation in that area. Alternatively, the arteriosclerotic plaque may increase its size (but not break) by decreasing the diameter or arterial lumen and occluding the artery and cutting the circulation in that area. In both cases, if this happens in the heart or brain, it could lead to death. Platelet activation is also critical for the development of thrombotic vascular occlusion, which is the most important pathophysiological mechanism leading to myocardial infarction or ischemic stroke (2). In addition to its important role in hemostasis, platelets also participate in inflammatory processes, angiogenesis and tumor progression (9) and they have been shown to play an important role in the progression of Alzheimer's disease (AD) (10, 11), which will be addressed in section 1.2.

CVD prevention strategies can be employed before a cardiovascular event occurs (primary prevention) or before further episodes take place (secondary prevention). Currently, in clinical practice, there are several drugs that act on different CVD manifestations (e.g. statins to lower cholesterol), but the search for new complementary pharmacological and/or dietary alternatives is still a priority.

1.1.1. Changes in eating habits to prevent CVD

The prevention of most CVD is performed by addressing behavioural risk factors such as tobacco use, poor diet, obesity, physical inactivity and abuse of alcohol, using population-wide strategies. Prevention, treatment, and control of these risk factors before clinical manifestation are, therefore, primary targets of public health interventions to reduce CVD (12).

An unhealthy diet constitutes a key factor in the initiation and progression of these diseases. Therefore, addressing suboptimal nutrition is of key prognostic relevance in primary and secondary prevention. Processed foods, high in sodium and hydrogenated fats, and low in fiber, are being often consumed in excess. These contemporary dietary changes have adversely affected global dietary parameters known to be related to health, resulting in an increase in obesity and chronic disease.

Since 1957, evidence that diet is important in the development of CVD is emerging. Those observations were pivotal in the development of the cholesterol hypothesis: dietary saturated fat increases serum cholesterol, which in turn leads to coronary atherosclerosis. In 1972, a joint statement from the National Academy of Sciences-National Research Council and the Council on Foods and Nutrition of the American Medical Association reported that the risk of coronary heart disease was linked to the blood cholesterol level (13).

There are several feeding studies regarding the effects of different dietary fatty acids on serum cholesterol levels, with predictive equations being developed (14). Several studies have suggested that different dietary patterns, like the Mediterranean diet, predict CVD or mortality (15). For example, diets rich in fruit, vegetables and low-fat dairy foods were reported to reduce blood pressure (BP) (16). In addition, increased consumption of omega-3 (n-3) fatty acids from fish oil or plant sources improve

endothelial dysfunction (17). In summary, it seems clear that changing to specific eating habits can confer great benefits regarding CVD prevention.

1.1.2. Biomarkers of CVD

'Biomarker' or 'biological marker' was first used in 1989, as a Medical Subject Heading (MeSH) term. This is defined as "measurable and quantifiable biological parameters which serve as indices for health and physiology" (18). It provides an avenue for research. The term "biomarkers" refers to a broad subcategory of medical signs, objective indications of medical state observed from in the patient, which can be measured accurately and reproducibly. They are measurement tools to determine disease progression and the effects of interventions and allow understanding the differences in clinical response that may be influenced by various variables (drugs, surgery, functional foods, etc.) (19). In terms of nutritional effects of selected food components, investigators are using a wide array of analytical tools to assess their actions on biological parameters related to CVD risk factors, such as hypertension, obesity, diabetes, and hypercholesterolemia.

1.1.2.1. Lipidic profile

In humans, an increase in total blood cholesterol and triglycerides (TG) levels is considered a key factor in the development of CVD. Hyperlipidemia or hypercholesterolemia are defined as elevations of fasting total cholesterol concentrations, which may or may not be associated with elevated TG concentrations. TG are not directly atherogenic, but represent an important biomarker of CVD because they are associated with atherogenic particles. Lipids are not soluble in plasma, but are instead transported in particles known as lipoproteins. High plasma LDL implies more substrate to be oxidized (ox-LDL) and form atheroma plaques (6). High-density

lipoproteins (HDL), instead, might be protective and are associated with decreased coronary heart risk, at least when they are effective on reverse cholesterol transfer (20).

Laboratory analysis of the lipid profile measures:

- Total cholesterol, which is the sum of different types of cholesterol.
- HDL cholesterol, which is usually called the "good" cholesterol by the lay public. High-density lipoproteins transport cholesterol back to the liver for elimination in a process called reverse cholesterol transport.
- LDL cholesterol, generally known by the lay public as the "bad" cholesterol. LDL lipoproteins that accumulate in the bloodstream can clog blood vessels (see the above-mentioned atherosclerosis) and increase the risk of heart disease.
- TG, which store energy until the body needs it. If the body accumulates too many triglycerides, the blood vessels can clog and cause health problems.

In Table 1, we can see the optimal, medium and high values, in human samples, of all the parameters mentioned above.

Table 1. Lipid profile measures (adapted from (21)).

LDL Cholesterol	<100	Optimal
	100-189	Normal
	≥ 190	High
Total Cholesterol	<200	Optimal
	200 – 239	Normal
	≥ 240	High
HDL Cholesterol	≥ 60	Optimal
	60-40	Normal
	< 40	High
TG	<150	Optimal
	150 – 199	Normal
	200-≥ 500	High

1.1.2.2. Markers of inflammation, oxidation, and endothelial function

Several inflammatory mediators, including adhesive and signaling mechanisms, are involved in early atherosclerotic lesion formation. Inflammatory processes involving activation of cytokines, enzymes, and growth factors continue to play a role in the plaques (22). Several circulating inflammatory mediators can be measured in plasma or urine samples. For example, high-sensitivity C-reactive protein (hsCRP) is a type of innate immune response protein. It is a non-specific inflammatory marker that has been studied in CVD and mediates atherosclerosis (23). hsCRP levels < 1 mg/L are desirable and reflect a lower atherosclerotic risk and a low systemic inflammatory status: levels between 1 and 3 mg/L indicate moderate vascular risk, and levels > 3 mg/L indicate higher vascular risk. Thromboxane B_2 (TXB_2) is an eicosanoid derived from cyclooxygenase-2 (COX-2), which can induce vasoconstriction and promote aggregations. There exists a strong relationship between elevated TXB_2 levels and risk of stroke, myocardial infarction and death. Tumor necrosis factor-alpha (TNFα) is synthesized by different cell types in response to physiological and pathophysiological stimuli such as inflammation (24), and plays an important role in CVD. Cytokines (different interleukins) are also good biomarkers of CVD, because they are highly expressed in the process of atheroma formation. Such cytokines are significantly associated with CVD risk, independently of obesity and conventional risk factors, in a log-linear manner (25). The chemokine MCP-1 plays a key role in a variety of pathophysiological processes in cardiovascular biology (26), being able to directly act on endothelial cells and to induce angiogenesis through the induction of vascular endothelial growth factor (VEGF). There are other markers that reflect lipid oxidation and participate in CVD progression, e.g ox-LDL and isoprostranes. Isoprostanes

(prostaglandins isomers) are a bioactive product of arachidonic acid metabolism and elevated levels of these molecules have been associated with several cardiovascular risk factors (27). The adipose tissue also plays an important role in CVD because it produces several proteins (adipocytokines) such as leptin, adiponectin, resistin, TNFα, and IL-6, that modulate insulin sensitivity (28). All these markers can be measured in plasma samples.

1.1.2.3. Glycemic profile

Levels of postprandial glucose and acute glucose levels may be good predictors of CVD. Control of glucose in diabetes patients was first proposed by Koenig and collaborators in 1976 (29). The metabolic abnormalities caused by diabetes mellitus induce vascular dysfunction and increase the risk of myocardial infarction, stroke, amputation, and death (30). Type 1 diabetes is due to insulin deficiency and type 2 diabetes is due to insulin resistance (IR). IR, or reduced insulin sensitivity, is a key pathophysiologic defect appearing too in CVD (31). The most commonly marker used to measure insulin resistance is the homeostatic model assessment (HOMA), which is derived from a mathematical calculation of the balance between hepatic glucose output and insulin secretion from fasting levels of glucose and insulin (32). HOMA is, among other risk factors, a reliable tool for predicting the risk of coronary events.

1.1.2.4. Anthropometric variables and vital signs

Anthropometric variables may be the best tools to screen and predict obesity and overweight, which are increasing in many countries. Abdominal adiposity has been considered one of the best predictors for CVD (33), and waist circumference (WC) and waist-to-hip ratio (WHR) have been widely used in epidemiological studies. Body weight (BW) is a quick and useful tool that is employed in nutritional studies. Body

mass index (BMI) is often used to reflect total body fat, and as surrogates for central body fat we often use the WC, WHR and waist to height ratio (WHtR) (34).

As for vital signs, observational studies have demonstrated associations between higher systolic blood pressure (SBP) and diastolic blood pressure (DBP), and increased CVD risk; the different reference values can be seen in Table 2. Moreover, high BP (or hypertension) directly increases the risk of coronary heart disease and stroke. High BP is a symptom that the arteries may have an increased resistance against the flow of blood, causing the heart to pump harder.

Table 2. Categories of BP in adults (adapted from (35)).

BP Category	**SBP**	**DBP**
Normal	<120 mm Hg	<80 mm Hg
Elevated	<120–129 mm Hg	<80 mm Hg
Hypertension		
Stage 1	130–139 mm Hg	80–89 mm Hg
Stage 2	≥140 mm Hg	≥90 mm Hg

1.2. Neurodegenerative diseases

In addition to CVD, one of the great challenges of our society - due to the aging of the population - are neurodegenerative diseases (ND). It is evident that age itself causes physiological changes that occur during normal brain aging and that can be exacerbated in vulnerable populations of neurons, initiating pathological processes that eventually lead to ND, especially AD. Neurodegeneration is defined as a progressive loss of structure, function and even death of neurons, affecting many of body's activities, such as balance, movement, talking, breathing, and heart function.

Depending on the type, ND can be serious or even life-threatening. Most of them have no cure and treatments may only help improve symptoms, relieve pain, and increase mobility. Many of these diseases have genetic origins, but sometimes the cause is unknown. According to the most recent Global Burden of Disease Study 2015, the burden of neurological disorders has increased substantially over the past 25 years because of expanding population numbers and aging (36). ND include: dementias, AD, amyotrophic lateral sclerosis, Friedreich's ataxia, Huntington's disease (HD), Lewy body disease, Parkinson's disease (PD) and spinal muscular atrophy. In this thesis, the focus was set on dementia, specifically in mild cognitive impairment (MCI) and AD.

The shortage of molecular data on aging is due to the difficulty of separating the effects of "age" from those of age-related diseases (atherosclerosis, diabetes, hypertension and dementia) in vascular function and cognition. Life expectancy increases significantly in developed countries and the prevalence of MCI, AD and other dementias increases with age (37, 38).

Age is the major risk factor for developing dementia, which is not an inevitable consequence of aging. Dementia not only affects the elderly: early onset dementia (defined as the onset of symptoms before age 65) represents up to 9% of cases. Some research has shown a relationship between the development of cognitive impairment and life-style related risk factors that are shared with other noncommunicable diseases.

Risk factors for the development of such diseases include physical inactivity, obesity, unbalanced diets, tobacco and harmful alcohol use, diabetes and hypertension in middle age, as well as depression, low educational attainment, social isolation and cognitive inactivity.

1.2.1. Mild cognitive impairment

The concept of mild cognitive impairment emerged at the end of the nineties as proposed by Ronald C. Petersen and is defined as an intermediate stage between normal cognitive aging and very early dementia (39). MCI patients mainly report memory impairment, especially episodic memory, disorders of language, attention and visual-spatial skills (40).

The prevalence of MCI is in the 14 to 18% range for individuals aged 70 years or older (41). The rate of conversion of MCI to AD is 10% to 15% per year and other types of dementia have been estimated at 5 to 16% for the elderly adult population (42, 43).

The causes of MCI are not yet completely understood; risk factors include aging, genetic (heredity) cause of AD or other dementia, and risk of CVD. Some of these risks factors cannot be altered, but others are modifiable such as cardiovascular factors, physical fitness and diet (44).

MCI exceeds what is normally expected for age, but does not meet the dementia criteria because functionality is preserved. There are no fixed events that determine the transition point between the asymptomatic and symptomatic phases of pre-dementia, or of the symptomatic pre-dementia phase at the beginning of dementia (45).

MCI can then be classificated in three subtypes (46):

- Amnestic: significant impairment of memory that does not meet criteria for dementia.
- Multidomain: mild deficiencies in multiple cognitive and behavioral domains.
- Non-amnesic monodomain: isolated impairment of some cognitive domain other than memory.

Despite efforts in the area of research to define the borderline between the normal and the pathological, it is known that, during aging, in the absence of neurological disease, there is a small decrease in the cognitive area, with visual impairment deteriorating first, while verbal skills and general knowledge are better preserved (47).

In structural neuroimaging studies, AD patients have a global cerebral volume loss, increased ventricular volumes, and atrophy in the hippocampal and the entorhinal cortex. Topographic gray matter loss correlates with very mild AD patients. MCI usually presents structural changes intermediate to those observed in AD, usually displaying mild, but significant, volume loss in specific brain regions, notably the hippocampal structures, and decreased cortical thickness (48). Both are important brain regions, which play complementary roles in learning and memory, and are clinically important in cognitive disorders (49).

Several studies have been oriented to the prediction of MCI conversion to dementia and an association between said conversion and high levels of microtubule-associated protein tau (TAU protein) has been suggested (see below) (50).

There are no effective pharmacological treatments available to treat MCI, although multidisciplinary strategies related to the prevention of chronic diseases associated with aging are being promoted. Both the Spanish and H2020 research programs include multidisciplinary lines with the aim of improving the understanding, prevention, early diagnosis and treatment of mental conditions and disorders of the elderly (Establishment of preventative strategies favouring the mental dimension of healthy ageing. Advancing active and healthy ageing: PHC-20-2014-solutions for independent living with cognitive impairment; PHC-21-2015-Early risk detection and intervention, etc.).

1.2.2. Alzheimer's disease

AD is the most common form of dementia and possibly contributes to 60–70% of all cases and affects 26 million people worldwide with an upward trend (51, 52). Each year, 4.6 million new cases are predicted, with numbers of affected nearly doubling every 20 years to reach 81.1 million by 2040 (53).

AD is a neurodegenerative disorder characterized by amyloid β-peptide (Aβ) deposits followed by neuron loss and decreased cognitive and memory habilities. Although it was characterized more than 100 years ago, little progress has been made towards an effective cure. AD starts slowly; first, it affects the parts of the brain that control thought, memory and language. People with AD may have difficulty remembering things that happened recently or the names of people they know. Over time, AD symptoms worsen: people may not recognize their relatives and may have difficulty speaking, reading or writing. They may forget how to brush their teeth or comb their hair. Later, they may become anxious or aggressive.

The etiology of AD is unclear. Largely, its pathophysiology is related to the death of neurons, initiating in the hippocampus, a crucial brain region involved in memory and learning; then, atrophy affects the entire brain. Indeed, AD is a polygenic and heterogeneous disorder with multiple patterns of expression. The late-onset sporadic form is the most prevalent, with the early-onset familial type being responsible for only about 1% of the cases. Within the risk factors we find nonmodifiable ones, like age and genetic factors, and modifiable factors such as life style (physical activity, diet, education), metabolic syndrome (diabetes, hypertension, obesity), and possibly depression (54).

The World Health Organization (WHO) states that AD develops in three stages:

- Early stage: the early stage of dementia is often overlooked, because the onset is gradual.
- Middle stage: as dementia progresses to the middle stage, the signs and symptoms become clearer and more restricting.
- Late stage: the late stage of dementia is one of near total dependence and inactivity. Memory disturbances are serious, and the physical signs and symptoms become more obvious.

Pathologically, AD is characterized by the presence of senile plaques that contain Aβ and neurofibrils of hyperphosphorylated TAU protein. This protein induces changes to the cellular architecture as well as to the localization and organization of subcellular organelles, in turn inducing synaptic interruption and apoptotic neuronal death (55).

The multifactorial etiology of AD makes the development of viable therapies difficult; current approved medications attenuate symptoms, but do not cure the disease. AD research has also had several failures in terms of developing disease-modifying therapies. Because this pathology begins many years before the symptoms appear, new scientific approaches now focus on the early stages of the disease, such as MCI.

1.2.3. Changes in eating habits to prevent ND

In recent years, growing evidence emerged from epidemiological studies indicates that certain nutritional deficiencies, especially in the elderly, significantly affect the processes of cognitive deterioration associated with aging (56).

Specific dietary patterns have been associated with increased risk of developing AD, whereas others are linked to protection. Other prospective studies showed that the Mediterranean diet, characterized by high consumption of cereals, fruit, vegetables,

legumes, and olive oil, is associated with lower incidence of AD and can improve cognition (57). The intake of unsaturated fatty acids (FAs) (both monounsaturated (MUFAs) and polyunsaturated fatty acids (PUFAs)) has been associated with improved cognitive performance and a decreased risk of age-related cognitive decline in long-term observational studies (58). The low intake of n-3 and phospholipids (PHLs), which are essential for the activity, functioning, and maintenance of the nervous system, is related to a greater risk of ND (59). Also, B-vitamins are important for neuronal functioning and cognition (60). n-3, mainly those of marine origin of long chain and high degree of unsaturation, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are involved in the prevention of CVD, improving cerebral vascularization in some neuro-psychiatric disorders, particularly in depression and in AD associated with aging (61).

For these reasons, specific dietary interventions or patterns might play preventive actions on dementia and its manifestations. Therefore, it is important to fully elucidate the role that dietary components (micro- and macronutrients) play at the molecular level, which enable to provide sound scientific advice to public health authorities.

1.2.4. Biomarkers of ND

Biomarkers are important to monitor the development of disease or for implementing evidence-based prevention. Mapstone et al. (62) reported for the first time that ten blood metabolites (consisting mainly of PHLs) could be used to predict future cognitive impairment in asymptomatic older adults. There is growing evidence from *in vitro* experiments, animal models, and observations in patients that links abnormal insulin signaling with AD. The underlying mechanisms include interference with the

metabolism of Aβ and TAU protein (63). Studies on brain pathogenesis propose Aβ, neuritic plaque (NP), and neurofibrillary tangle (NFT) aggregation. However, autopsies of brains of aged people who were not diagnosed with a neurological disease, also revealed Aβ, NFT, Lewy bodies, inclusions, synaptic dystrophy, and loss of neurons and brain volume. In short, other processes can contribute to ND (64).

These data highlight the enormous potential that biomarkers have to predict the shift of cognitive deterioration, in a preclinical phase, to AD.

1.2.4.1. Glycemic profile

Epidemiological evidence indicates that diabetes is a risk factor for AD onset and development (65, 66). Longitudinal epidemiologic studies show that diabetes increases AD risk by 50–100%; this includes type 2 diabetes, which accounts for 90% of all cases (67). Lower glucose metabolism has also been observed in MCI (68). In 1979, the glucose tolerance test was introduced, as an intermediate state in the transition in glucose homeostasis from normal to diabetes state (69). IR-associated risk factors such as obesity, poor diet, physical inactivity, aging, and genetic predispositions are correlated with cognitive dysfunction. Overall, the analysis of plasma biochemical biomarkers related to diabetes provides relevant information in ND studies.

1.2.4.2. Mitochondrial biogenesis

Growing evidence suggests that mitochondrial dysfunction plays an important role in the aging of the brain and in the pathogenesis of ND (70). This loss of cognition associated with age is seen in both animal and human models. The underlying mechanisms that lead to endothelial dysfunction and dementia are poorly understood. However, as mentioned, all these damaging changes are probably linked to mitochondrial dysfunction and, in particular, to mitochondrial oxidative decay, which is

an important contributor to aging. Although the loss of mitochondrial function associated with age is undoubtedly multifactorial, several lines of evidence indicate that certain molecular and cellular alterations are significantly involved in the progression of the changes that ultimately lead to an altered mitochondrial energy metabolism (71).

It has been hypothesized that the characteristics of mitochondrial deterioration are symptomatic of a vicious circle where the defective electron transport chain (ETC) complexes contribute to a greater generation of reactive oxygen species (ROS) by the mitochondria, which in turn increases the damage and mutations of the mitochondrial DNA (mtDNA), and finally affects reciprocally the structure of ETC components (72). Damages and deletions in mtDNA that correlate with lower age-related oxidative respiratory activity have been reported in the liver and heart muscle of rats, the liver and skeletal muscle of primates, and in the skeletal and cardiac muscle of human beings (73). Ultimately, higher rates of ROS production increase the oxidative modification of membrane lipids and proteins, to the point where the accumulation of molecular defects exceeds the capacity to maintain mitochondrial homeostasis through autophagy (74). Mutations in genes that encode mitochondrial proteins could compromise the mitochondria by altering the components of ETC, resulting in an inefficient transport of electrons, decrese adenosine triphosphate (ATP) and an increase in the production of superoxide. The oxidative damage resulting from the mitochondria can compromise its ability to satisfy the demands of the brain's equilibrium energy. The accumulation of oxidative damage to mitochondria, proteins and nucleic acid in the brain can lead to neuronal and cognitive dysfunction (75).

Because mitochondrial dysfunction plays an important role in ND, obtaining information on the mitochondrial energetic state is obviously pertinent.

1.2.4.3. Signaling related to ND

Insulin signaling

A relationship between the appearance of cognitive disorders and IR has been observed, and the latter has been identified as a major risk factor for the onset of AD (76). Research shows that cerebral glucose metabolism impairment, in diferents brain regions, precedes the onset of dementia and can predict decline from normal cognition to MCI and AD. Hippocampus and temporal cortex show the highest levels of insulin receptor expression, indicating the important role of insulin in learning and memory (77). At the cellular level, it has been seen that astrocytes are responsible for converting glucose into lactate and transfer it to neurons, which is an important neuronal energy reservoir during periods of high activity, like learning and memory (78).

Insulin is a polypeptide hormone produced and secreted by the beta cells of the islets of Langerhans, reaching the brain through the cerebrospinal fluid and crossing the blood-brain barrier. Once insulin reaches the cells it binds to its receptor, which induces transphosphorylation, giving rise to an activation loop, activating the insulin receptor substrate (IRS) and, in turn, the signaling pathway of the phospho-inositol-3-kinase (PI3K) and/or the pathway of the mitogen-activated protein kinases (MAPK). The insulin-signaling pathway (Figure 1) is interrupted in IR because regulatory mechanisms are altered. In AD, the IRS-1 is hyperphosphorylated (79). Given all the above, exploring the levels of relevant actors involved in these pathways provides important information when conducting ND-related studies.

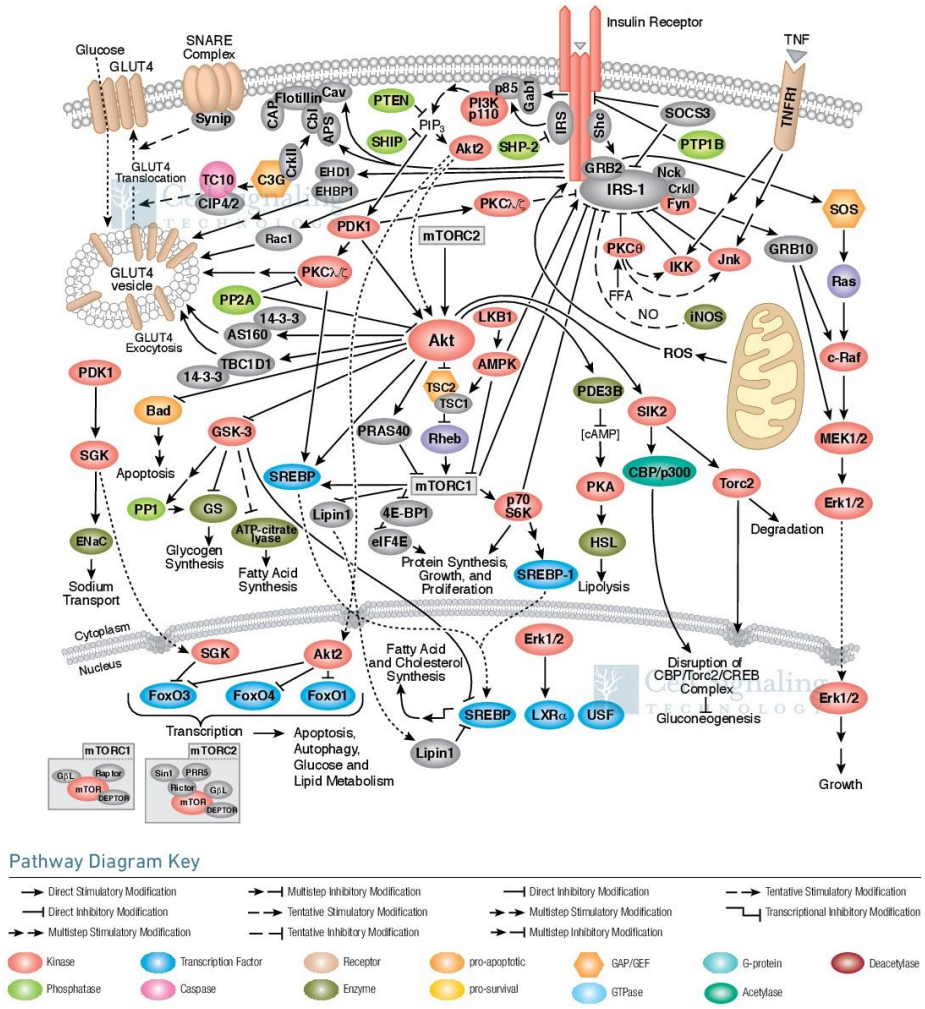

Figure 1. Insulin signaling. Image taken from https://www.cellsignal.com

Neurotransmission

Impulse transmission from one neuron to another depends on the action of neurotransmitters through synapses. Synaptic transmission is the biological process by which a neuron communicates with a target cell through a synapse. There are two types of synaptic transmission, chemical and electrical. Their chemistry involves the release of a neurotransmitter from the pre-synaptic neuron and its consequent binding to specific post-synaptic receptors. The electrical synapse involves the transfer of electrical signals through gap junctions (80). During neurodegeneration, a progressive loss of

structure or function of neurons occurs, which can end with their death (81). Synaptic dysfunction is an early change in both cognitive decline and AD (82).

Studying genes that code for pre- and post-synaptic proteins and their proteins, in key tissues, provides information on how nerve cells function and the possible evolution that they can have towards ND. This information is more robustly correlated with cognitive decline than neuropathological hallmarks of AD (83). For example, α-synuclein and other synaptic proteins have been shown to be differentially expressed in various ND (84).

2. Functional foods and micronutrients

Many studies have investigated the health benefits of various functional food ingredients, including n-3 FAs, polyphenols, fiber, and plant sterols, among others. These bioactive compounds may help to prevent and reduce the incidence of chronic diseases. Several epidemiological studies clearly show that proper diets are associated with lower incidence of degenerative diseases, namely cardiometabolic disorders (CMD) and cancer (85). Neurodegeneration also appears to be partially preventable by following specific dietary regimens. The general consensus is that plant foods should provide the major portion of caloric intake, as this reduces the development of certain diseases, in particular cancer and CVD. In the past few years, there has been a steadily growing interest in the role that oligonutrients (aka micronutrients), commonly found in plant foods, might play in the maintenance of human health. Although "plant oligonutrient" deficiencies in diets are yet to be described, the recent discovery that several phenolic molecules, namely catechols, exert interesting biological activities led to a re-evaluation of the contribution of these compounds to human health (86). This is also leading to increasing marketing of "functional foods" or "nutraceuticals", i.e.

fortified or enriched foodstuffs that are attributed health-promoting effects. The rationale on which the hypothesis that certain phytochemicals might contribute to lowering CVD and ND is based on the notion that oxidative and inflammatory processes play a role in the onset and development of these pathologies (87, 88). Several phytochemicals (notably catechols) are endowed with enzyme-modulating activities, hence expressing the potential to lower cardiovascular risk. It is noteworthy that a diet that derives a large proportion of its calories from plant foods allows having a significant daily intake of these phytochemicals. Although the absorption, metabolism, and excretion of most of these compounds is being slowly elucidated, such high intake makes this group of molecules particularly interesting from a preventive medicine point of view, and the study of their bioavailability is very important (89). Also, it is important to note that, apart from phytochemicals, the human body cannot synthesize lipid- and water-soluble vitamins that, therefore, must also be taken up from the diet or provided through functional foods or "pharmaceutical" preparations.

Epidemiologically, the best diets (in terms of cardio- and chemoprevention) are the Mediterranean and the Japanese ones (90). Of note, there is growing evidence that classic risk factors for CMD such as high serum cholesterol and blood pressure are not much different in the populations of Southern Europe and Japan, as compared to other Western countries. This suggests that other unexplored risk factors (for which, however, biochemical evidence is accumulating), in addition to the classic ones, may be favorably affected by these diets (91). Food composition studies have shown that both diets are rich in minor components derived from fruits, vegetables, tea, red wine, olive oil, and n-3, derived from fish, nuts, certain seed oils, and vegetables. The typical Mediterranean and Japanese diets also provide adequate intakes of fiber and folic acid. Fiber intake (both of cereal and of fruit/vegetable origin) has been shown to reduce the incidence of

all the clinical events related to atherosclerotic disease. Adequate intakes of folic acid, on the other hand, are associated with reduced blood levels of homocysteine, an amino acid that damages the inner arterial lining (endothelium) and whose high blood concentrations have been associated with elevated cardiovascular risk. Finally, consumption of complex carbohydrates, as bread, pasta, and rice (largely represented in the Mediterranean and Japanese diets) is also associated with favorable health effects. For instance, they induce a limited post-prandial rise of plasma glucose and consequently a limited insulin response, thus reducing the risk of developing overweight and diabetes (two important risk factors of CVD and ND).

Though the healthful properties of the Mediterranean diet as a whole have gained recognition, basic researchers are nowadays concentrating their efforts on individual food items (e.g. cereals, fruits, vegetable, olive oil) and their components (e.g. fibers, vitamins, polyphenols). Why so? There are several reasons to investigate the molecular effects of single food components. Observational claims resulting from epidemiological studies often fail when tested in randomized trials. Epidemiology and observational associations are prone to fallacies because they most often rely on food frequency questionnaires. In this sense, a recent analysis showed that, in the National Health and Nutrition Examination Survey, the energy intake measures inferred from the questionnaires of two thirds of the participants are incompatible with life (92).

The best way to obtain reliable data for diets and their components would be to carry out randomized controlled trials (RCTs), as is customary in pharmacological research. With the notable exception of the PREDIMED (93), this proves to be nearly impossible because the effects of diets are – by definition – modest and difficult to gauge clinically and because the optimal setting is that of a metabolic ward where participants are required to stay for prolonged periods of time. In the absence of

controlled conditions such as those of metabolic wards, it becomes very difficult to assess compliance. Indeed, the field of metabolomics is addressing precisely this, i.e. finding circulating or urinary metabolites indicative of selected foods. Budgetary and technical issues currently prevent the extensive application of metabolomics to human studies (94).

One alternative way to provide supportive evidence in favor or against dietary profiles is to build enough *in vitro* and animal data to back claims and strengthen dietary guidelines. By targeting relevant enzymatic systems and cellular pathways, researchers can shed light on the effects of food components and, in turn, substantiate claims. Of course, this approach requires rigorous scientific behavior and the use of relevant concentrations or doses, appropriate biomarkers, and adequate exposures. Interestingly, human trials with selected, isolated food components (or mixtures of them) can be carried out to test the effects of biochemical and physiological responses (95).

In summary, by singling out the contribution of micronutrients to the protective activities, one can better focus dietary guidelines and, when possible, formulate appropriate functional foods or nutraceuticals.

Indeed, an emerging area of research situates itself at the interface of pharmacology and nutrition. This field is currently experiencing renewed impetus as several food components are being employed as medicines, either directly or as pro-drugs. As a matter of fact, there are areas in which the border between food and “pharma” is not well-defined, as the former often contains several bioactive compounds including secondary plant molecules (polyphenols), fibers, friendly bacteria, essential FAs, probiotics, and other contributions. Furthermore, several current drugs are derived from natural products including those to which humans have been exposed via diet. Indeed, it is sometimes difficult to distinguish between bioactive molecules termed

"drugs" and other substances classified as "nutrients". Optimal health and prevention of chronic diseases can be attained (to a certain extent) by modulating the intake of macro- and micro-nutrients, often in pharmacological doses as in the case of supplements, nutraceuticals, and functional foods. Classic pharmacotherapy can also be accompanied by adjunct treatments with nutrition-derived remedies that are often able to decrease the doses of medicines and/or lessen their side effects. In summary, the border between pharma and food is becoming less distinct (96).

A healthy lifestyle, with exercise and a varied and balanced diet can prevent and even reduce the risk of developing certain types of diseases such as CVD and ND.

3. Nutrigenomics tools

It should be noted that in the last decade, omics tools have allowed deepening the knowledge of the metabolism of some diseases where genetic techniques are not enough. These techniques allow determining possible biomarkers that have become increasingly important in the understanding of many diseases such as CVD and ND.

Nutrigenomics attempts to study the genome-wide influences of nutrition. It investigates the impact of dietary components on the genome, the proteome and the metabolome (the sum of all genes, proteins, and metabolites), studies the effect of different diets or foods in specific cells, tissues and organisms, and how nutrition influences homeostasis (97). Furthermore, nutrigenomics aims to identify the genes that influence the risk of diet-related diseases on a genome-wide scale, and to understand the mechanisms that underlie these genetic predispositions.

Dietary intervention studies have successfully used transcriptomics and proteomics to show how diet induces alterations in gene and protein expression for three different purposes: 1) provide information about the mechanism underlying the effect of

a certain nutrient, 2) determine possible biomarkers, and 3) identify pathways regulated by micronutrients.

Thanks to all these techniques, we can better understand how the diet or different foods influence CVD and ND.

The main nutrigenomics tools used during this PhD work mainly concerned transcriptomics and proteomics:

1) Transcriptomics

Microarrays

This omics tool, important in transcriptomics, quantitatively measures levels of gene expression. It consists of different nucleic acid known probes that are chemically hybridized to a substrate, which can be a microchip or a glass slide. The analysis of samples from nutritionally relevant studies in animal and cell models have led to the identification of genes regulated at the messenger RNA (mRNA) level by exposure to different dietary components in different tissues (98). The adipose tissue, for example, is important to study lipid metabolism, because it plays an important role in obesity and CVD (99). Likewise, the intestine, which is crucial in regulating cholesterol and lipid homeostasis in the body, is the limiting tissue of lipid absorption and it participates in the elimination of cholesterol through faeces in the process called trans-intestinal cholesterol excretion (TICE) (100).

With regards to ND, many learning and memory processes are influenced by gene transcriptional changes and are affected by nutrition (101). In this sense, the studying of brain tissues such as the hippocampus and temporal cortex are of special relevance, due to their involvement in memory formation and because they suffer great damage and morphological changes during neurodegeneration (102).

RT-qPCR

This technique is used to amplify and quantify gene expression. From a sample of ribonucleic acid (RNA), retrotranscription (RT) is carried out, using the reverse retrotranscriptase enzyme, to convert the RNA into DNA complementary (cDNA), then real-time polymerase chain reaction (PCR) is performed in the same way as conventional PCR, a deoxyribonucleic acid (DNA) template, dNTPs (deoxynucleotide triphosphates), at least a pair of specific primers, and a thermostable DNA polymerase; a fluorophore is added to the mixture and the emitted fluorescence is measured in the thermocycler. The PCR process usually consists of a series of temperature changes that are repeated 25-40 times, called cycles. This reaction is usually divided in three stages: the first, at high temperatures to activate the enzyme and to dehybridize both cDNA chains; the second, at a temperature around 50-60 °C, allows the hybridation between the primers and the template DNA; and the third, at 68-72 °C, facilitates the polymerization by the DNA polymerase.

RT-qPCR is commonly applied after microarrays analysis, in order to confirm and validate the results obtained from the latter (103). Besides mRNA quantification, other types of non-coding RNA, like microRNA (miRNAs), can also be analyzed with this tool (104). Another use of this technique is through the design of specific primers for known genes, within the same signaling pathway, or involved in some process of interest, for example involved in neurotransmission, to study ND, or involved for example in the expression of phase II enzymes, to study antioxidants' functions or detoxifications.

Next generation sequence (NGS)

The first technique used to sequence is Sanger sequencing, based on the chain termination method that Frederick Sanger and colleagues developed in 1977 (105). It was widely used since it was created until 2007. This method has been replaced by NGS, which refers to high-throughput DNA sequencing (seq) technologies. Millions or billions of DNA strands can be sequenced in parallel, yielding substantially more throughput and minimizing the need for the fragment-cloning methods that are often used in Sanger sequencing of genomes (106).

The applications of this technique are:

- Sequencing and assembly of de novo genomes
- Re-sequencing of genomes and analysis of variability Analysis of the functional activity of the genome and epigenetics

-Chromatin analysis: ChIPseq, FAIREseq: regulation of gene expression

- Epigenetic analysis (Bisulphite seq) DNA methylation

- Transcriptome gene expression analysis: RNAseq

- Metagenomics studies

A large number of NGS platforms exist, including Illumina, the Applied Biosystems SOLiD System, 454 Life Sciences (Roche), Helicos HeliScope, Complete Genomics, Pacific Biosciences PacBio and Life Technologies Ion Torrent (107).

Understanding the transcriptome throught RNA Seq is essential for the interpretation of the functional elements of the genome and for understanding the molecular constituents of cells and tissue.

Recent advances in the RNA-Seq workflow, from sample preparation to library construction to data analysis, have enabled researchers to further elucidate the

functional complexity of the transcription(108). The main advantage of RNA-seq with the previous methods is the high performance, the sensitivity and the ability to discover new transcripts, gene models and non-coding RNA, such as miRNAs. This technique enables a deep profiling of the transcriptome and, in the case of this thesis, the opportunity to elucidate transcryptional responses to different diet components.

2**) Proteomics**

Proteomics is the large-scale study of different proteins in a sample, cell line, tissue, or organism. This term was first coined in 1995 (109). The proteome is not constant, it differs from cell to cell and changes over time. To some degree, the proteome reflects the underlying transcriptome. However, protein activity (often assessed by the reaction rate of the processes in which the protein is involved) is also modulated by many factors in addition to the expression level of the relevant gene. In dietary context, proteomics is the study of proteins and their functions and how they are modulated in response to different foods. Proteomics can provide significant biological information such as:

- Which proteins interact with a particular protein of interest
- Which proteins are localized to a subcellular compartment (e.g. the effect of food in different organs).
- Which proteins are involved in a biological process

Mass-spectrometry (MS)-based Super-SILAC

Mass-spectrometry (MS) based quantitative proteomics are the most commonly applied techniques besides Tandem-MS and gel-based techniques such as differential in-gel electrophoresis (DIGE). These high-throughput technologies generate huge

amounts of data. Databases are critical for recording and carefully storing the data. MS is a powerful method that allows identifying the compilation of proteins expressed in biological systems, in addition to their interactions and modifications. Stable isotope labeling with amino acids in cell culture (SILAC) has risen as a powerful quantification technique in MS. Specifically, the super-SILAC technique uses a mixture of SILAC-labeled cells on study as a spike-in standard for accurate quantification of unlabeled samples, for the quantification of human or animal tissue samples (110).

Western Blot

Western blotting is an important technique used in cell and molecular biology. By using a western blot, researchers are able to identify specific proteins from a complex mixture of proteins extracted from cells or tissues. After protein extraction and quantification, the same concentration for each sample is loaded onto polyacrylamide gels and submitted to Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE). In SDS-PAGE, proteins are separated based on their molecular weight. Proteins are then transferred to a solid support (i.e. membrane) and, finally, the proteins of interest are visualized by marking with proper primary and secondary antibodies (111). This technique is commonly used to study proteins known to be involved in signaling pathways of interest or for the validation of results found in the mass spectrometry.

4. miRNAs as biomarkers

miRNAs are short (-22 nucleotides) noncoding RNA molecules acting as post-transcriptional regulators, mainly as gene expression repressors, but also as activators (112). Generally, under physiological conditions they work as "buffers" of gene expression by regulating different genes at the same time. Thus, a single miRNA can act

on hundreds of mRNAs, modulating the regulation of specific biological processes acting in a coordinated manner in pathways of functionally related genes (113).

miRNAs biogenesis begins with a large RNA precursor, pri-miRNA, which is transcribed and matured in the nucleus by the RNA polymerase II. RNase III and protein partner Drosha subsequently cleave it near the hairpin stem base. The pre-miRNA precursor has a hairpin of 60 to 70 nucleotides and is released in the cytoplasm by exportin-5. Through DICER protein, it is divided, resulting in a double stranded miRNA/miRNA with 21–25 base pairs, which is unwound by RNA induced silencing complex (RISC). RISC will then carry the mature miRNA to target messenger RNAs resulting in gene silencing. Its function is to regulate a series of cellular processes, such as apoptosis, differentiation, cell cycle, and immune functions (114). However, under stress conditions, its function is enhanced, revealing its importance in health and illness, and it can be altered by a wide range of environmental factors such as smoking, alcohol, sleep, exercise, stress, radiation and nutrition.

Numerous pathways affected by miRNA regulation, such as lipid metabolism, glucose homeostasis, vascular integrity and endothelial cell function, are involved in CVD (115). The role of some miRNAs in lipid metabolism, in tissues other than the intestine has been described, but it is still unknown whether miRNAs may be actively participating in the regulation of lipid metabolism in the gut. There is scientific evidence indicating that some components of our diet can have beneficial effects through the modulation of the expression of miRNAs (116). However, very few studies have evaluated such effects in depth.

miRNAs are present, stable, and detectable in the circulation. Several cardiac miRNAs are detectable in blood and might be employed to improve CVD diagnosis

(117). Dimmeler et al., in a pioneering work, observed changes in miRNA levels in the serum of patients with coronary artery disease compared to controls (118). After this study, numerous miRNAs have been reported to be involved in CVD. Examples include miRNAs implicated in cardiovascular death (miRNA-132, 140 and 210), in acute heart failure (miRNA-423), or in cardiac autophagy (miRNA-22) (119-121).

To determine whether some diet components modulate the expression of important miRNAs involved in disease, it is important to search for their modulation or/and to demonstrate whether the therapeutic inhibition of those miRNAs could have benefits *in vivo* (using animal models). As proof of concept, the therapeutic inhibition of miRNA-122, using antisense oligonucleotides, has been shown in clinical trials (122).

Current approaches for ND identification and progression include the analysis of proteins in cerebrospinal fluid (CSF), biomarkers in blood samples, and various imaging techniques. Minimally-invasive specific biomarkers of ND would facilitate patient selection and disease progression and some groups have proposed the use of circulating miRNAs for disease detection (123, 124). For example, the expression of miRNA-9 was reported to be downregulated in the blood serum of AD patients compared with age-matched controls (125). Other miRNAs reported to be dysregulated in AD are miR-125b and miR-138, showing increased levels in the hippocampal CA1 region (126). Moreover, miRNAs play essential roles in synaptic plasticity by regulating the expression synthesis of relevant proteins to the synapse (127).

5. Micronutrients studied

5.1. Soy Isoflavones

Soy provides various nutrients, such as proteins, FAs and isoflavones. Soy consumption might be beneficial in terms of reducing the concentration of sugars in the blood (important against diabetes) (128), prevent osteoporosis by reducing female estrogens, and protect against CVD (129). Soy isoflavones (SI) have estrogenic activity, due to their structural similarity with 17-β-estradiol. SI are different from human endogenous steroids, yet have the ability to bind to estrogen receptors (130). The major isoflavones in soybean are genistein and daidzein.

Isoflavones are used as supplements. It is suggested that daily consumption of ≥25 g of soy protein, with its associated intact phytochemicals, can improve lipid profiles in humans with hypercholesterolemia. The European Food Safety Authority (EFSA) has conducted a risk assessment of SI supplements, with intakes up to 150 mg/day by post-menopausal women, and no adverse effects were found (131). Indeed, the supposed cardioprotective effects of soy have been attributed mainly to its protein components (132), but also notable examples include genistein, daidzein, and glycitein (133, 134). Numerous clinical studies claim benefits of genistein and daidzein in the chemoprevention of breast cancer and prostate cancer, cardiovascular disease, and osteoporosis (135). For example, the intake of soy isoflavones from one month up to one year could significantly improve serum lipid profiles in both healthy and hypercholesterolemic individuals (136). It has also been reported to improve vascular function and lower blood pressure (137).

However, the belief that soy foods and their supplements are beneficial in human health is increasingly controversial due, precisely, to the estrogenic activities of this micronutrient, which could negatively affect the risk of breast cancer, in particularly among ER-positive breast cancer survivors (135). Moreover, a meta-analysis of 10

randomized placebo-controlled trials, where plasma lipoproteins concentrations had been determined, revealed no significant effect of soy isoflavones treatments (138).

In summary, the effects of SI on CVD and its associated risk factors have not been fully clarified and further investigation is needed.

5.2. Hydroxytyrosol

Hydroxytyrosol (HT) is a compound almost exclusively found in olives and in extra virgin olive oil. It is generated by the hydrolysis of oleuropein (OE), which occurs during the maturation of the fruit and during the process of producing table olives, giving rise to OE aglycone, hydroxytyrosol, and elenoic acid.

HT - which can currently be synthesized (unlike OE) - has properties that have aroused great interest for its use as a potential nutritional supplement and as a preservative in cosmetics as well as in the nutraceutical and food industry. Its short-term exploitation at the industrial level is envisioned.

There are numerous studies that have shown that HT has numerous antioxidant properties, beneficial effects on the cardiovascular system, as well as antimicrobial, anticarcinogenic, anti-inflammatory and neuroprotective properties (139).

Regarding the safety of hydroxytyrosol, the first acute toxicity test with HT in experimental animals was carried out in 2001. Results showed that a single dose of 2 g/kg of body weight did not produce any adverse effect, other than for piloerection (disappeared after 48 hours), or macroscopic alteration of internal organs (140). Subsequent studies evaluating acute toxicity in rats, such as the 90-day chronic toxicity test, confirmed the absence of toxic effects at doses of 2000 mg/kg/day, as well as the absence of teratogenic and mutagenic effects (141). Moreover, in 2013, a study

proposed a No Observed Adverse Effect Level (NOAEL) of 500 mg/kg BW/day for HT, which would represent for humans (considering a safety factor of 100) 5 mg/kg BW/ day of HT or 300 mg/day of HT for a person of 60 kg (142). Finally, HT has been recently approved by the EFSA as a novel food (143).

At the molecular level, HT is able to modulate several enzymatic activities related to CVD, including the inhibition of platelet aggregation (144) and pro-inflammatory enzymes, such as the enzyme 5-lipoxygenase (145, 146), and the stimulation of the inducible form of nitric oxide synthase (147). The effects of olive leaf extracts at the vascular level have been demonstrated in a rabbit model (148), but the relevance of these studies in humans is not known. While most data have been obtained *in vitro*, several experimental evidence has been confirmed *in vivo*. Regarding human studies, more than 15 experiments have been conducted with healthy volunteers or patients and the vast majority confirm that olive oil rich in phenols positively modifies indirect markers of cardiovascular disease; other studies compared the effect of olive oil with those of extra virgin olive oil. Of note, extra virgin olive oil contains phenols other than HT (149). Therefore, conducting studies in humans with this compound, as the only phenolic source, is vital in order to determine the role it plays in CVD and neurodegenerative disorders. HT and other related olive phenols have been indeed tested as supplements in humans, and to date there have been only two studies with HT, as the only source of polyphenols (150, 151). The most notable results are the inhibition of the generation of thromboxane B_2 in whole blood, suggesting an antithrombotic activity *in vivo* (152, 153). Regarding neurodegeneration, epidemiological studies report a better cognitive function in people (mostly elderly) whose diet includes a large part of olive oil. Studies showed that the Mediterranean diet is associated with lower incidence of AD and improved cognition (57, 154). HT is being explored in neurodegeneration

and ameliorates AD-involved neuronal impairment via modulating mitochondrial oxidative stress, neuronal inflammation, and apoptosis (155, 156).

5.3. Bioactive polar lipids

Lipids are a group of organic molecules that fulfill numerous biological functions in the body such as energy reservation (TG), structural PHLs, sphingolipids (SLs), cholesterol), regulation (steroid hormones), and transport (bile acids) (157). Within lipids, polar lipids (PLs) are characterized by being amphipathic molecules in which an apolar region and a polar region are identifiable. Among the PLs, we find the PHLs and the SLs.

PHLs are compounds with potent biological activities and are the main lipid constituents of all biomembranes and especially those of the nervous system. In addition to their structural action, they also regulate biological processes such as intracellular signaling processes, many of which are altered in various metabolic and neurological diseases (158). PHLs, depending on their vegetable or animal origin, predominantly contain an unsaturated FAs in position sn-2, such as oleic, linoleic, arachidonic or EPA or DHA (of marine origin), while the sn-1 position predominantly carries a saturated FAs, such as stearic or palmitic. The phosphate group, bound by a phosphodiester bond, gives the name to PHLs, depending if it is choline (phosphatidylcholine, (PC)), ethanolamine (phosphatidylethanolamine, (PE)), inositol (phosphatidylinositol, (PI)) or serine (phosphatidylserine, (PS)). In the case of SLs, the alcohol sphingosine binds to a FA resulting in sphingomyelin (SM).

The absorption of diet FAs is much higher when they are part of PLs, since in the intestine the latter are absorbed almost completely (> 90%). It has been documented that almost 20% of PLs are absorbed in the intestine passively, without hydrolyzation,

and that they are preferably incorporated into HDL, from which they are transferred to plasma and reach the membranes as such or as their corresponding isoforms, thus contributing to the renewal of the PHLs of cell membranes. These mechanisms are highly complex and have not been fully elucidated (159).

An important source of polar lipids of dairy origin is the milk fat globule membrane (MFGM), mainly composed of triacylglycerides and different concentrations of other compounds such as diacylglycerides, monoacylglycerides and cholesterol. MFGM also contains different phospho- and sphingolipids. Buttermilk (BM), a by-product obtained from butter manufacturing, contains high proportions of PS and SM. PS and SM acquire special importance due to their activities in the organism. PS plays an important role in cellular functions including the integrity of the mitochondrial membrane, release of presynaptic neurotransmitters, activity of postsynaptic receptors and activation of the protein Kinase C in the formation of memory (160). The decrease in SM-containing myelin content in the brain has been related to the slowing down of the cognitive process associated with aging (161). Another important source of polar lipids from marine origin is krill oil (KO). Krill (*Euphausia superba*) is a small marine crustacean mainly found in the Antarctic Ocean. It is rich in long chain polyunsaturated n-3 as well as in PHLs. KO is a unique source of EPA and DHA, mostly incorporated in PC. Numerous studies in animals have shown the importance of PC in the diet of pregnant mothers because of its impact on brain development and function (162).

The EFSA recently concluded that n-3 effectively reduce TG levels when consumed at intakes of 2 to 4 g/day. Food and Agriculture Organization (FAO) and WHO recommend a daily intake of 500 mg of EPA + DHA (163).

Both BM and KO, because of their composition, could be helpful against ND.

HYPOTHESIS & OBJECTIVES

Cardiovascular and neurodegenerative diseases are the leading cause of mortality and morbidity worldwide. Environmental factors such as stage of development, nutrition, physical exercise, environmental pollution, among others, might modulate (in either direction) the onset and development of degenerative diseases. Despite progress in the prevention and treatment of both maladies, there is a clear need to identify novel biomarkers and pharma-nutritional interventions to further ameliorate prognosis. In other words, the search for new pharmacological or dietary therapeutic alternatives for CVD and ND prevention or treatment must, and does so, continue.

The hypothesis of this doctoral thesis is that selected micronutrients can partially protect against neurodegenerative and/or cardiovascular diseases. Further, we investigated the specific molecular mechanisms of action of these selected molecules.

The main objective of this doctoral thesis is that, by studying the biological effects of certain micronutrients, we can contribute in the laying of scientific basis for their potential use to prevent, treat, or at least mitigate the consequences of these pathologies. In this sense, we aimed to study the nutrigenomic and epigenetic effects of certain micronutrients on the expression of key genes and proteins included in pathways relevant to cardiovascular and/or neurodegenerative diseases, through *in vitro* and *in vivo* (experimental animals and human clinical trials) approaches. In addition, we aimed at investigating the beneficial actions of these compounds, to identify novel biomarkers of their effects, and to look for new molecular targets, in order to provide strong scientific evidence to their potential incorporation into nutraceuticals or functional foods.

The specific aims are listed below and sub-divided into three chapters, based on the micronutrients under study.

Chapter 1: Soy isoflavones (Objectives 1)

- To investigate the nutrigenomic actions of soy isoflavones (in nutritionally relevant amounts) in mice, with a specific focus on adipose tissue, due to their fundamental role in cardiovascular metabolism.

Chapter 2: Hydroxytyrosol (Objective 2)

- Determine the effect of hydroxytyrosol supplementation in healthy volunteers, investigating its bioavailibility, as well as its potential effect on the modulation of Phase II enzymes and certain microRNAs in PBMCs. In order to achieve this, a pilot randomized, crossover, placebo-controlled trial was conducted.
- Determine the molecular mechanisms by which hydroxytyrosol exerts its possible beneficial effects against cardiovascular diseases and to search for potential new therapeutic targets of this micronutrient, using *in vitro* and *in vivo* (rodents) models.
- Analyze the beneficial effects of hydroxytyrosol in an *in vitro* model of Alzheimer's disease.

Chapter 3: Bioactive phospholipids (Objective 3)

- To investigate the potential beneficial effects of bioactive phospholipids on glucose metabolism and mitochondrial dysfunction, in aged rats.
- To study the effects of bioactive phospholipids on cognitive functions and to look for cerebral microRNAs as potential markers in the treatment of mild cognitive impairment, in aged rats.

COMPOUNDS USED & WORKPLAN

To carry out the work performed, during this PhD thesis, three different compounds were used:

SOY ISOFLAVONES

To perform the work reported in Chapter 1 we used a mixture of glycosylated soy isoflavones, with genistein and daidzein as the main ingredients (14.7%; Soyselect®), kindly donated by Indena (Milan, Italy). Soyselect® is a standardized extract obtained from soy with a double standardization procedure and which contains 13%–17% of isoflavone glycosides genistein and daidzein and ~ 18% of B-group saponins, as quantified by HPLC. The product is prepared by extracting ripe whole soybeans or oil-free soy flour with aliphatic alcohols through a patented process of industrial manufacture (US Pat. Nos. 6,280,777 and 6,617,757). One gram of extract also contains 0.058 g of protein, 0.035 g of fat and 0.023 g of ash. The batch (nr. 30432/M1) used in this study for the preparation of the feed, contained specifically 14.7% isoflavone glycosides and 21.2% B-group saponins.

The feed was prepared by the company Research Diets (New Jersey, USA), that developed the control diet and the one supplemented with soy isoflavones. The amounts used in the diet correspond to a supplementation of 0.45 g% of the extract that is a final quantity of isoflavones mix in the diet corresponding to ~0.661 mg/g of solid diet (0.0661%). Both the supplemented and the control diet provided equal amounts of 24.0 Kcal % from proteins, 15.0 Kcal % from fats, and 61.0 Kcal % from carbohydrates.

To fulfil Objective 1, the following working scheme was followed (Figure 2).

Figure 2. Workplan to investigate the nutrigenomic actions of soy isoflavones.

HYDROXYTYROSOL

To perform the work described in Chapter 2, hydroxytyrosol was supplied as described below.

Hydroxytyrosol used in the human clinical trial

For the double-blind, randomized, placebo-controlled trial, we gave subjects encapsulated Hytolive® with different doses of hydroxytyrosol. These were kindly donated by Genosa I+D (Madrid, Spain). Hytolive® is an aqueous extract of olive mill wastewater produced as a standardized syrup or dry powder. Both the syrup and the powder have the characteristic odor of processed olives. The biologically important constituent of this extract is hydroxytyrosol. The constituents of Hytolive® are also found in olive oil, as well as in table olives and are, therefore, commonly consumed by humans. In the process of obtaining this product, no solvents or other chemicals are used.

The Hytolive® we used in the human study contains 10-15% of HT and has been officially recognized by the American FDA (Food and Drug Administration) as safe for its food use in humans, acquiring the GRAS status (generally regarded as safe).

The doses used in this study were 5 or 25 mg per day, against placebo. The clinical trial work outline and the different doses used are represented in Figure 3.

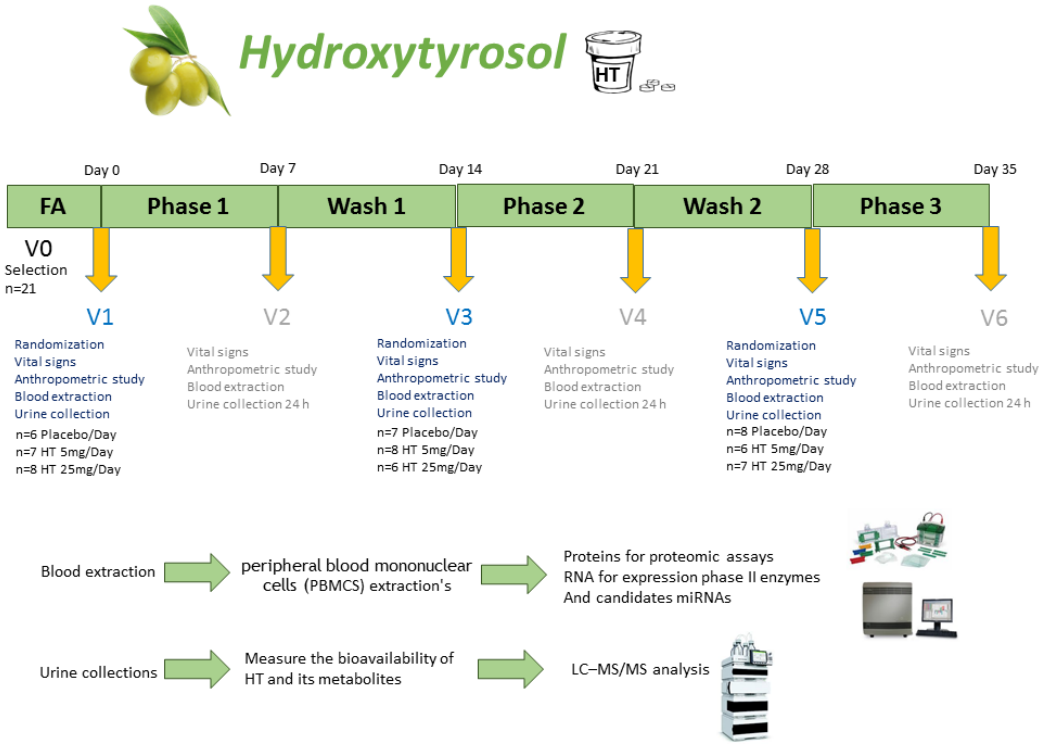

Figure 3. Work scheme of the pilot study to determine different effects of supplementation with hydroxytyrosol in healthy volunteers.

Hydroxytyrosol used in studies with laboratory mice

In the investigations with mice, pure hydroxytyrosol, kindly donated by Seprox Biotech (Madrid, Spain), was used. Two experimental designs were realized. In the long-term feeding study, hydroxytyrosol was incorporated in the chow provided by Research Diets (New Jersey, USA). As in the research with soy isoflavones, the company provided both the control diet and the diet supplemented with 0.03 g% HT. This dose closely approximates human intake and is a very low one once body surface area is taken into account. Each diet provided 24.0%, 15.0% and 61.0% kcal from protein, fat and carbohydrates, respectively. For the acute ingestion studies, mice were

given, by gavage, 15 mg of HT dissolved in water. The scheme of the working plan is detailed in figure 4.

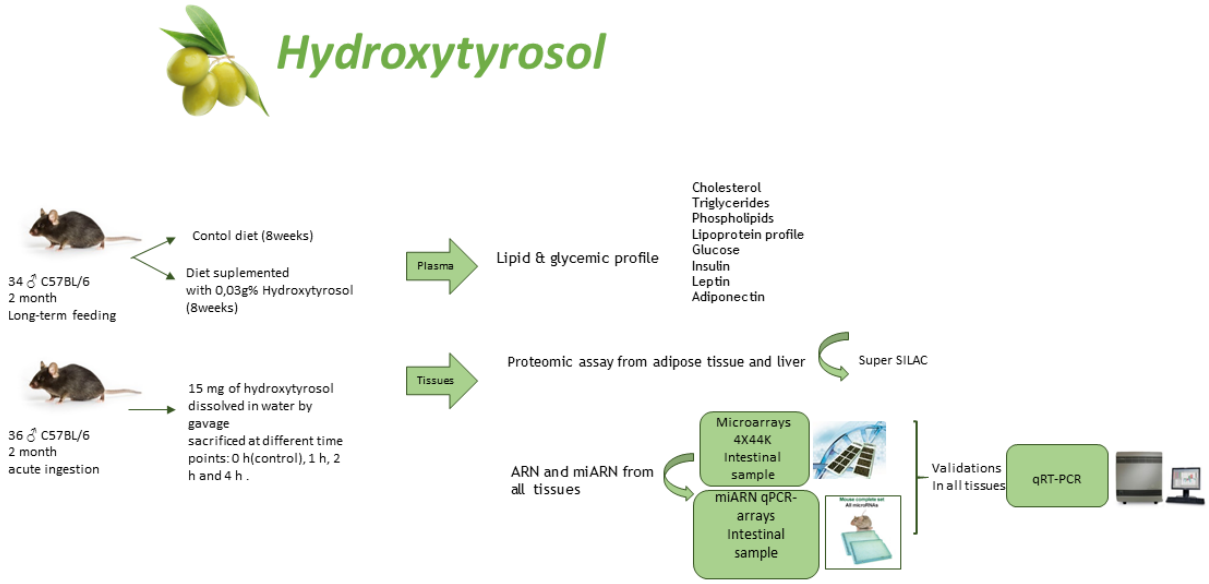

Figure 4. Workplan of the in vivo studies with hydroxytyrosol described in objective 2.

Hydroxytyrosol in cell culture studies

For *in vitro* studies, pure hydroxytyrosol (99.5%, tested by HPLC; batch E012 0814172327, donated by Seprox Biotech (Madrid, Spain)) was used.

First, 1 g of the compound was resuspended in 6.48 ml of absolute ethanol to achieve a stock concentration of 1 M. This was done in a glass tube, to prevent the compound from adhering to plastic. Compound manipulation was always done in darkness to avoid hydroxytyrosol oxidation.

This stock solution was used at different concentrations depending on the experimental design carried out and taking into account the physiological concentrations required in each experiment.

The working scheme and the different concentrations used in the *in vitro* studies is described in Figure 5.

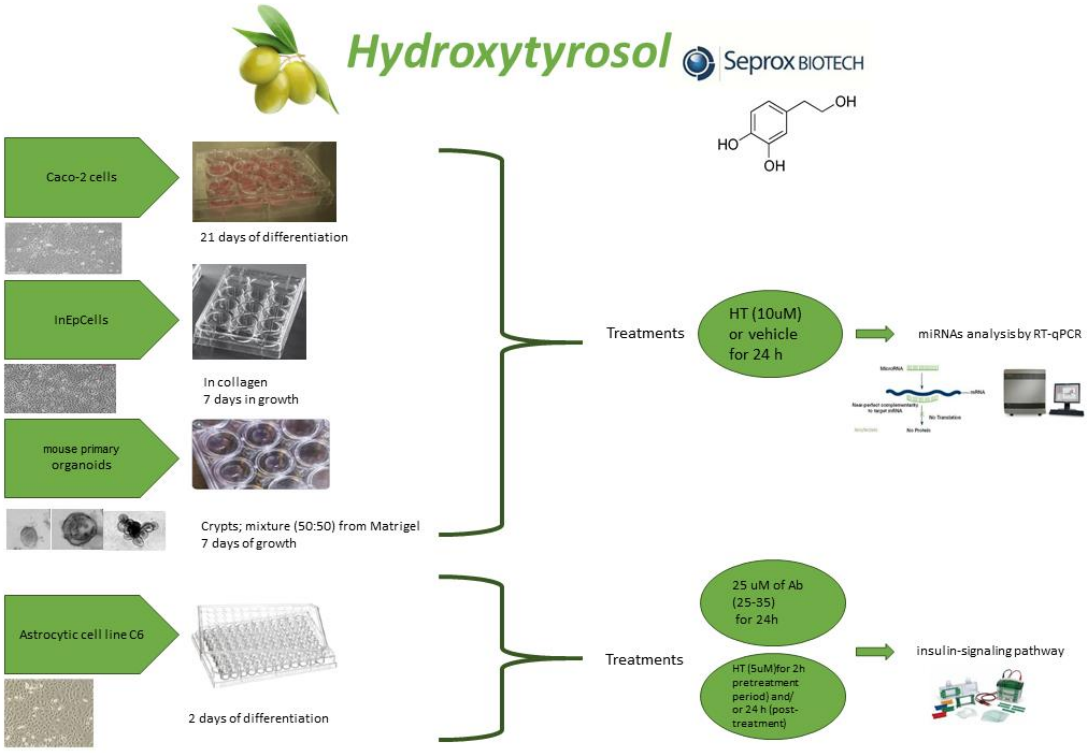

Figure 5. Workplan of the *in vitro* investigations with hydroxytyrosol described in objective 2.

POLAR LIPIDS FROM MILK (BM) & MARINE ORIGINS (KO)

BM powder and Antarctic KO from *Euphausia superba* were used to elaborate the compounds used in the experiments described in Chapter 3, regarding the potential beneficial effects of PLs against MCI. Both were kindly donated by Reny Picot (Oviedo, Spain) and AKO3™ (Aker Biomarine Antarctic AS, Oslo, Norway), respectively.

The elaboration of the compounds used was carried out by Dr. Javier Fontecha and his team members, Pilar Castro Gomez and Alba García Serrano, at the Research Institute of Food Science **(**CIAL) in Madrid, Spain. BM fat was extracted by pressurized liquid extraction (PLE) method using an accelerated solid extraction ASE-200 extractor (Dionex Corp. Sunnyvale, CA) starting from 15g of the product that was mixed with sea

sand (1:1) and loaded into a stainless steel extraction cell. To obtain the maximum BM fat yield, the extraction was based in the optimized PLE method by Castro-Gómez *et al (164).*

In brief, the concentration of the PLs of both compounds was increased as follows. BM was dissolved with acetone in a ratio of 1:5 (v/v). The mixture was kept on a stirrer or and subsequently centrifuged. The precipitate obtained was subjected to the same process by incorporating more acetone in a 1: 2 (v/v) ratio. Finally, the latter precipitate, namely butter milk fat concentrate (BMFC), was evaporated and stored at -35 °C for further use in the preparation of rats' feed.

KO was dissolved in 96% (v/v) ethanol in a 1:8 ratio and kept for 24 h at different temperatures. Then, it was evaporated and the final precipitate, krill oil concentrate (KOC), was used for the elaboration of animal feed.

The animals were fed a standard feed from LabDiet (EURodent diet 22%) along with a daily single-dose supplementation of the study concentrates in the form of strawberry jellies. The monodosis was prepared for 4 groups: refined olive oil as the control, BMFC, KOC, and BMFC+KOC mixture. Concentrations and experimental design are described in Figure 6.

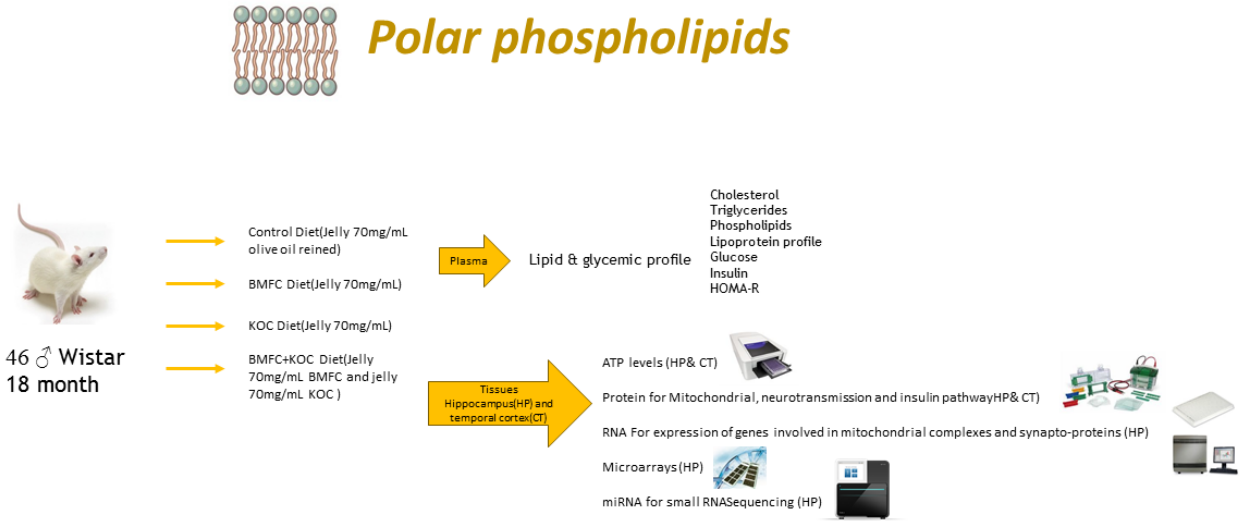

Figure 6. Work scheme of the research involving polar phospholipids supplementation described in objective 3.

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RESULTS AND DISCUSSION

Chapter 1. *Soy isoflavones*

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Soy isoflavones in nutritionally relevant amounts have varied nutrigenomic effects on adipose tissue.

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Article

Soy Isoflavones in Nutritionally Relevant Amounts Have Varied Nutrigenomic Effects on Adipose Tissue

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Abstract: Soy consumption has been suggested to afford protection from cardiovascular disease (CVD). Indeed, accumulated albeit controversial evidence suggests that daily consumption of ≥25 g of soy protein with its associated phytochemicals intact can improve lipid profiles in hypercholesterolemic humans. However, the belief that soy foods and supplements positively impact human health has become increasingly controversial among the general public because of the reported estrogenic activities of soy isoflavones. In this study, we investigated the nutrigenomic actions of soy isoflavones (in nutritionally-relevant amounts) with a specific focus on the adipose tissue, due to its pivotal role in cardiometabolism. Young C57BL/6 mice were maintained for eight weeks under two different diet regimes: (1) purified control diet; or (2) purified control diet supplemented with 0.45 g% soybean dry purified extract (a genistein/daidzein mix). Soy isoflavones increased plasma total cholesterol concentrations and decreased triglyceride ones. Circulating leptin levels was also increased by soy consumption. Differentially expressed

genes in adipose tissue were classified according to their role(s) in cellular or metabolic pathways. Our data show that soy isoflavones, administered in nutritionally-relevant amounts, have diverse nutrigenomic effects on adipose tissue. Taking into account the moderate average exposure to such molecules, their impact on cardiovascular health needs to be further investigated to resolve the issue of whether soy consumption does indeed increase or decrease cardiovascular risk.

Keywords: soy; isoflavones; leptin; genistein; daidzein

1. Introduction

Soy consumption has been suggested to afford protection from cardiovascular disease (CVD) [1]. This notion largely stems from observation that CVD mortality rates are lower in Asian countries, where soy is an important part of the diet. Indeed, accumulated evidence suggests that daily consumption of ≥25 g of soy protein with its associated phytochemicals intact can improve lipid profiles in hypercholesterolemic humans, even though this has not been confirmed yet [2,3].

The purported cardioprotective effects of soy have been mostly attributed to its proteic constituents [4], yet soy also contains isoflavones, *i.e.*, phytoestrogens with potent estrogenic activity; notable examples include genistein, daidzein, and glycitein [5,6]. Numerous clinical studies claim benefits of genistein and daidzein in chemoprevention of breast and prostate cancer, cardiovascular disease, and osteoporosis as well as in relieving postmenopausal symptoms [7]. However, the belief that soy foods and supplements positively impact human health is becoming increasingly controversial among the lay public. This is due precisely to the estrogenic activities of soy isoflavones, which might negatively impact breast cancer risk, in particular among ER-positive breast cancer survivors [7].

In short, the effects of soy and its component on CVD and associated risk factors have not been fully elucidated. In this study, we investigated the nutrigenomic actions of soy isoflavones (in nutritionally-relevant amounts) with specific focus on the adipose tissue, due to its pivotal role in cardiometabolism.

2. Results and Discussion

2.1. Body Weight, Food Intake and Lipid Profiles

To investigate whether chronic feeding of soybean extract had an effect on body parameters, we measured body intake and food intake of C57BL/6 male mice maintained for eight weeks on either a purified control diet or control diet supplemented with 0.45% soybean extract, which is equivalent to 0.0661% of isoflavone mixture pure aglycones. No changes in such parameters were observed (Table 1A), but soy isoflavone supplementation induced a significant increase in plasma cholesterol concentrations (Table 1B) as compared with control diet. Conversely, triacylglycerol concentrations were significantly reduced.

Table 1. Body weight and food intake (**A**) and cholesterol and triglyceride circulating concentrations (**B**) in controls and in mice fed a 0.45% Soyselect® diet.

(A)			
Body Weight		**Food Intake**	
g		g/day	
Control diet	29.1 ± 2.1	**Control diet**	3.0 ± 0.1
0.45% Soyselect®	27.7 ± 3.5	**0.45% Soyselect®**	2.7 ± 0.1
(B)			
Cholesterol		**Triglycerides**	
mg/dL plasma		mg/dL plasma	
Control diet	117.5 ± 9.3	**Control diet**	67.6 ± 16.1
0.45% Soyselect®	132.5 ± 2.9 *	**0.45% Soyselect®**	53.7 ± 8.14 *

Notes: Values are means ± SD; $n = 7$. Body weight, cholesterol, and triglyceride data are those obtained at the end of the study. Food intake data are those measured at the beginning and at the end of the study. * $p < 0.05$ as compared with controls.

2.2. Leptin Concentrations

Feeding 0.45% Soyselect® led to a significant increase in circulating leptin concentrations which rose from 1.49 ± 0.27 to 3.85 ± 1.16 ng/mL (Figure 1).

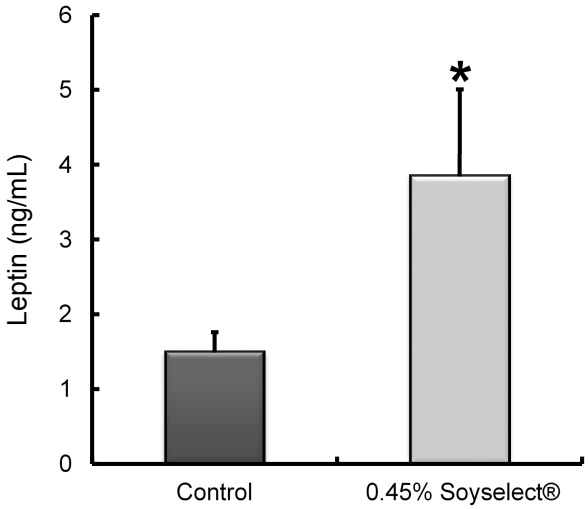

Figure 1. Leptin circulating concentrations in mice receiving either chow diet (control) or 0.45% Soyselect® during eight weeks ($n = 7$ per group). * $p < 0.05$ as compared with controls.

2.3. Peritoneal Fat Genome Expression Profiles

Whole-transcriptome microarray analysis was carried out on perigonadal adipose tissue samples isolated from mice fed either purified control diet or 0.45% soybean extract diet. A total of 983 genes (437 downregulated and 546 upregulated) were differentially expressed at (a 2-fold cutoff with a q value of less than 5%). Complete lists of upregulated and downregulated genes are shown in Supplementary Tables S2 and S3, respectively.

GENECODIS3 was used to perform biological pathway enrichment analyses of all downregulated genes in the interaction network (Table 2). The results show that a wide range of genes involved in many different biological pathways are modulated by Soyselect®. We selected three significantly enriched molecular functions, which include MAPK signaling, chemokine signaling, and TGFβ signaling, because they play pivotal roles in several cellular processes such as adipocyte differentiation and proliferation [8]. Specifically, a Venn diagram was constructed to evaluate the number of shared genes these three pathways: MAPK signaling [six genes], chemokine signaling [four genes], and TGFβ signaling [two genes] (Figure 2). The diagram shows that three genes, *i.e.*, MAPK1 (mitogen—activated protein kinase 1), PRKCB (protein kinase c, beta), and HRAS1 (Harvey rat sarcoma virus oncogene) are shared by the MAPK and chemokine groups; one gene, *i.e.*, MAPK1 is shared by all three groups and one gene, *i.e.*, ROCK1 (Rho-associated, coiled-coil containing protein kinase 1) is shared by chemokine and TGFβ signaling groups.

Table 2. Gene co-occurence annotations found by GeneCodis (molecular function) for genes downregulated by Soyselect® in perigonadal white adipose tissue.

Genes	NGR	TNGR	NG	TNG	Hyp	Hyp *	Annotations
53 genes	1999	37681	53	365	3.21184×10^{-11}	4.5287×10^{-9}	GO:0000166: nucleotide binding (MF)
42 genes	1501	37681	42	365	9.08868×10^{-10}	6.40752×10^{-8}	GO:0016787: hydrolase activity (MF)
38 genes	1314	37681	38	365	2.51857×10^{-9}	1.18373×10^{-7}	GO:0005524: ATP binding (MF) GO:0000166: nucleotide binding (MF)
39 genes	1421	37681	39	365	6.44848×10^{-9}	2.27309×10^{-7}	GO:0005524: ATP binding (MF)
61 genes	2999	37681	61	365	2.77158×10^{-8}	7.81585×10^{-7}	GO:0005515: protein binding (MF)

p-values have been obtained through hypergeometric analysis (Hyp) corrected by FDR method (Hyp *) NGR, number of annotated genes in the reference list; NG, number of annotated genes in the input list.

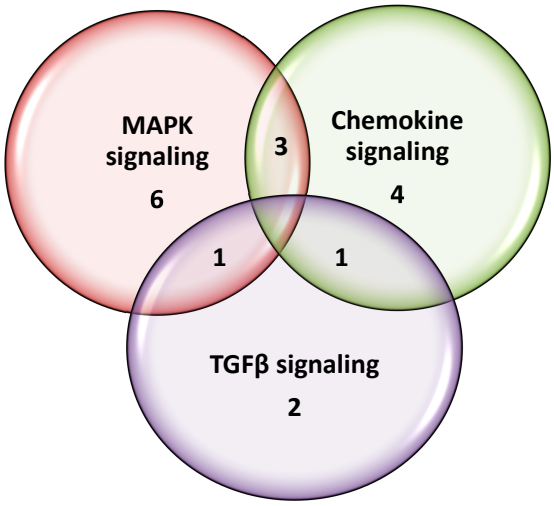

Figure 2. Venn diagram of selected pathway analyses. Pathway analyses of Soyselect®-modulated genes were performed and MAPK, TGFβ, and chemokine signaling were included as examples.

2.4. Gene Expression Validation by qRT-PCR

MAPK, TGFβ and chemokine signaling selected genes were analyzed by qRT-PCR (Figure 3) for validation. We also report other known genes related to lipid metabolism in adipose tissue and other highly modulated genes that came out from microarrays analysis, using a customized RT^2 profiler PCR array (Supplementary Table S4).

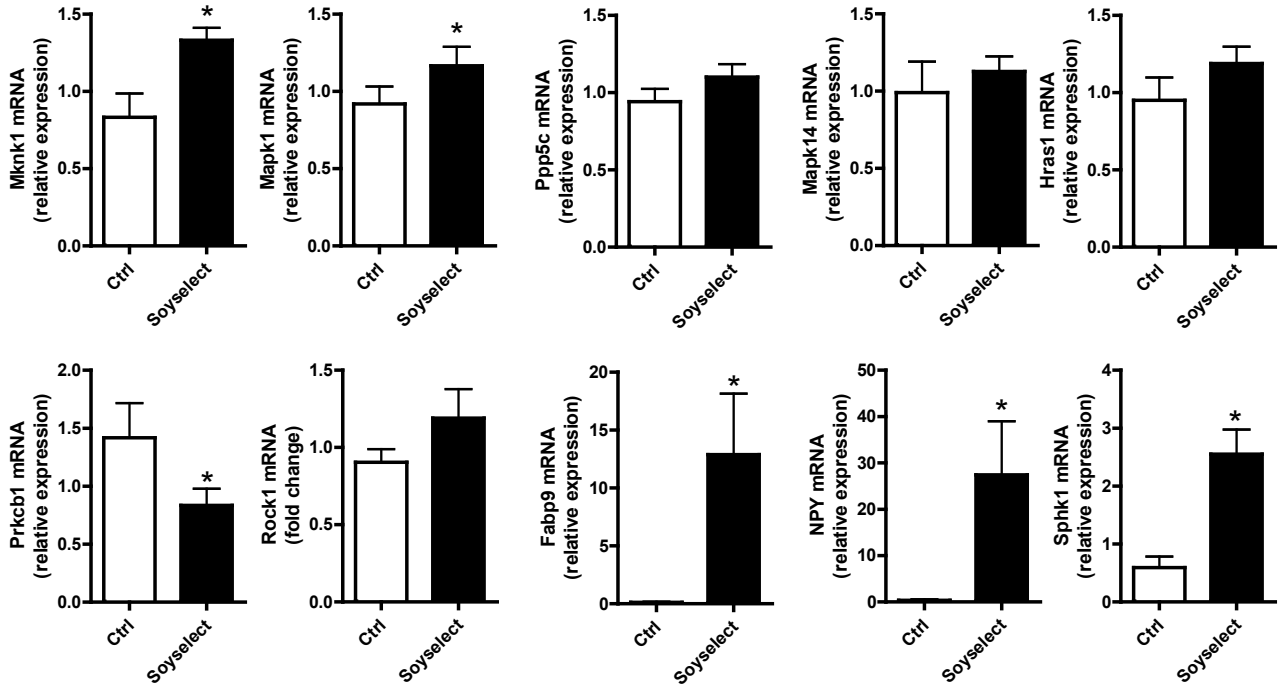

Figure 3. RT-qPCR validation of selected genes related to MAPK, TGFβ, chemokine signaling, and others related to lipid metabolism. Mice received either chow diet (ctrl) or 0.45% Soyselect® during eight weeks. mRNA is given as relative expression (n = 6 per group). * $p < 0.05$ as compared with controls.

Only Mknk1, Mapk1, and Prkcb1 were found to be modulated by the genistein/daidzein mix, while the other genes were not confirmed by qRT-PCR, suggesting either a modest effect of Soyselect® on adipose tissue gene expression or lack of sensitivity of microarray experiments, which highlight the need for validation using qRT-PCR. However, other genes related to adipose biology and lipid metabolism were found to be modulated by Soyselect®, including the fatty acid binding protein 9 (Fabp9), neuropeptide Y (Npy), and sphingosine kinase 1 (Sphk1). In most cases, validated genes exhibited a more modest effect in their expression when evaluated by qRT-PCR than by microarrays.

2.5. Discussion

We have performed the first nutrigenomic study of soy isoflavones in adipose tissue. Our data show that these molecules, administered in nutritionally-relevant amounts [6,7,9], have diverse albeit modest effects on adipose tissue, which is an organ prominently involved in cardiometabolism [4]. As mentioned, the low cardiovascular mortality of some Asian countries, particularly Japan, has often been attributed to the high consumption of soy products, e.g., miso, natto, shoyu, soy beans, *etc.* [3,5].

Inadequate epidemiological research prevents from drawing firm conclusions [10] and it is noteworthy that, while most of the cardioprotective effects of soy have been ascribed to its proteic fraction [4,11,12], these products only contribute small amounts of protein to the whole diet. Therefore, the real contribution of soy protein to cardioprotection has been questioned [2]. In addition to putatively healthful proteins, soy is rich in isoflavones such as genistein and daidzein. These compounds are the subject of active research and current recommendations on their consumption are under intense scrutiny. In fact, isoflavones resemble 17-β-estradiol in structure, and as such are able to bind the estrogen receptor (ER) *in vitro*, behaving much as a natural selective estrogen receptor modulator (SERM) [13]. Therefore, soy isoflavones might act as tumor-promoting or tumor-inhibiting agents, likely depending on cell type, dose, and genetic predisposition. As far as the cardiovascular system is concerned, the current consensus appears to be that estrogens exert complicated and poorly understood effects on cardiovascular health [14,15]. When the effects of estrogens are analyzed for men and women separately, they clearly exert a protective effect on cardiovascular function during a woman's childbearing years [14]. The challenge is now to convey these beneficial effects without unwanted steroid side effects on other organs and tissues, e.g., the breast [14]. Finally, genistein and daidzein have also been suggested to be neuroprotective and myorelaxant, namely in the detrusor muscle [16].

In adipose tissue, genistein and daidzein upregulate genes related to MAPK (Figures 2 and 3) and downregulate chemokine signaling gene Prkcb1 (Figure 3). The MAPK family is attracting considerable attention because of its vast implications in signaling and crosstalk with other signaling networks [17]. Indeed, some authors are suggesting considering the possibility of targeting MAPK-mitochondria interactions in the prevention and treatment of heart disease [17]. Some polyphenols for which cardioprotective properties have been suggested—such as epigallocatechin gallate—do increase MAPK [18]. Yet, as recently reviewed by Hopkins [19], MAPK generally have strong prosurvival effects on macrophages and can have varied effects on scavenger receptor expression and foam cell formation. The resulting effects on atherosclerosis can be nuanced and difficult to predict [19]. Chemokines are expressed vessel wall cells and emigrated leukocytes. These molecules play important roles in atherosclerotic vascular disease, where they exert various functions including cell recruitment [20]. The actions of chemokines in vascular inflammation are stimulating research for therapeutic agents aimed at these molecules. In addition to the vascular district, chemokines play important roles in the infarcted heart, where unrestrained inflammation induces matrix degradation and cardiomyocyte apoptosis [21]. Consequently, inhibition of pro-inflammatory signals may be effective in patients with defective resolution of postinfarction inflammation. Of note, a recent publication reported that isoflavone supplementation induced anti-inflammatory gene expression in equol postmenopausal producers [22].

We also measured some circulating surrogate markers of cardiovascular disease. Notably, we recorded significantly increase circulating leptin concentrations in mice fed with soy isoflavones, namely Soyselect® as compared with controls (Table 2). Even though the extent and precise nature of leptin's contribution to cardiovascular disease is still unclear [23], accumulated evidence points to its negative role in vascular impairment and hypertension. It is worth noting that leptin can promote angiogenesis and induce neovascularization. In addition, high blood concentrations of leptin associated with obesity can lead to arterial endothelial dysfunctions, impaired arterial distensibility, and proliferation and migration of vascular smooth muscle cells. In humans, greater soy consumption

appears to be associated with a lower presence of elevated total cholesterol, dyslipidemia, hyperuricemia and fewer cardiometabolic disturbances components [24]. No significant effect of soy isoflavones on blood pressure and endothelial molecules has been recorded [9,25]. A meta-analysis also reported that soy isoflavones have an effect of lowering blood pressure in hypertensive subjects, but not in normotensive subjects [26]. In short, the effects of soy isoflavones on the vasculature and blood pressure appear to be minimal in humans.

Soy isoflavones also increased total cholesterol while lowering triacylglycerol concentrations. The true human relevance of these findings is equivocal because, even though hypercholesterolemia is a known risk factor for CVD, lower triacylglycerols' concentrations are also associated with better CV prognosis. It is noteworthy that cholesterol circulates in mice almost exclusively as high-density (HDL)-c [27]. As soy consumption brings about hypolipidemic effects [27], the true significance of the observed cholesterol increase is equivocal and deserves further investigation. We found that Fabp9, Npy, and Sphk1 were upregulated by Soyselect® (Figure 3). Fabp9, Npy and Sphk1 are genes that regulate different aspects of lipid metabolism [28], adiposity [29], or adipocyte lipolysis [30]. Whether these and other Soyselect®-regulated genes are responsible for the observed effects on plasma cholesterol and triglycerides levels is not known and needs to be further investigated.

In conclusion, we add further evidence to the notion that soy isoflavones have assorted effects (both positive and negative) on cardiometabolic risk factors [31–33]. Keeping into account the moderate average exposure to such molecules, their impact on cardiovascular health need to be further investigated to solve the issue of whether soy consumption does indeed increase or decrease cardiovascular risk.

3. Experimental Section

3.1. Materials

A soybean purified extract containing isoflavones glycosides genistein and daidzein (14.7%; Soyselect®), was kindly donated by Indena (Milan, Italy). Soyselect® is a standardized extract obtained from soy with a double standardization procedure and which contains 13%–17% of isoflavone glycosides genistein and daidzein and <18% of B-group saponins, as quantified by HPLC [34,35]. The product is prepared by extracting ripe whole soy beans or oil-free soy flour with aliphatic alcohols through an industrial manufacturing proprietary process [36,37]. One gram of extract also contains 0.058 g of protein, 0.035 g of fat, and 0.023 g of ash, with the remaining matter undefined (Supplementary Table S1). The batch (nr. 30432/M1) used in this study contained 14.7% isoflavone glycosides and 21.2% B-group saponins. Of note, saponins increase isoflavone's bioavailability (unpublished data). SuperScript III First-Strand Synthesis System for RT-PCR was from Invitrogen (Madrid, Spain). Qiazol was from Qiagen (Izasa, Barcelona, Spain).

3.2. Animals and Diets

This investigation conforms to the Guide for the Care and Use of Laboratory Animals, published by the US National Research Council [38] and was approved by the Animal Experimentation Committee of the Universidad Complutense de Madrid.

Young C57BL/6 mice (2 months old, $n = 14$, *i.e.*, seven mice for each diet) were acclimatized in the animal facility on a 12:12 light/dark cycle, with the period of darkness between 7:00 a.m. and 7:00 p.m., for at least one week before experimentation. During this time, animals were fed a standard chow diet; food and water were given *ad libitum*. Then, mice were maintained for eight weeks under two different diet regimens (Research Diets, Inc. New Brunswick, NJ, USA): (1) purified control diet or (2) purified control diet supplemented with 0.45 g% soybean dry purified extract (genistein/daidzein mix). The final quantities of isoflavones mix in the diet correspond to ~0.661 mg/g of solid diet (0.0661%). Each diet provided 24.0%, 15.0%, and 61.0% kcalories from protein, fat, and carbohydrates, respectively. Their detailed composition is given in Table 3. To reduce diurnal variations, animals were sacrificed between 10:00 and 11:00 a.m., after an overnight fast. Mice were anesthetized with isoflurane and a midline incision was cut in the abdomen. Blood samples were collected from the vena cava. Heparin (0.4 mg/mL) was injected by means of the iliac vein and Hank's balanced salt solution (HBSS; pH 7.4) was perfused through the portal vein for 2 min to remove blood. Tissues were quickly removed, washed twice in ice-cold HBSS, and snap-frozen and stored at −80 °C. In order to verify the dietary effects of soybean extract, body weight and food intake have been evaluated.

Table 3. Composition of the experimental diets.

	Control		**Soyselect®**	
	g%	Kcal%	g%	Kcal%
Protein	23	24	23	24
Carbohydrate	60	61	60	61
Fat	6	15	6	15
Ingredient	**g/kg diet**			
Casein	244		244	
L-Cystein	3		3	
Corn Starch	318		318	
Maltodextrin 10	45		45	
Dextrose	250		250	
Cellulose	75		75	
Inulin	25		25	
Sunflower Oil	29.5		29.5	
Olive Oil	18.6		18.6	
Lard	18.5		18.5	
Mineral Mix S10026	10		10	
Dicalcium Phosphate	13		13	
Calcium Carbonate	5.5		16.5	
Potassium Citrate	5.5		16.5	
Vitamin Mix V10001	10		10	
Retinyl Acetate, 500,000 IU/g	0.048		0.048	
Choline Bitartrate	2		2	
Genistein/Daidzein mix	0		4.5	
Cholesterol	0.146		0.146	
Total	1083.84		1088.34	

3.3. Determination of Circulating Leptin Concentrations

Plasma concentrations of leptin were determined by ELISA kit, according to the manufacturer's instructions (Mouse Leptin, 96-well plate assay, Millipore, Madrid, Spain).

3.4. Determination of Plasma Lipid Concentrations

Plasma cholesterol concentrations were determined by the Amplex Red cholesterol assay kit (Invitrogen, Madrid, Spain) following the manufacturer's instructions. Plasma triglycerides were determined using an L-type triglyceride M test kit, according to the manufacturer's instruction (Wako Chemicals, Neuss, Germany).

3.5. RNA Isolation and Analysis

Total RNA from perigonadal adipose tissue (100 mg) was isolated using Qiazol Lysis Reagent (Qiagen, Isaza, Barcelona, Spain) and QIAGEN RNeasy Mini kit columns (Qiagen). RNA was quantified using a NanoDrop-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Madrid, Spain) and purity was assessed by measuring the ratio of absorbance at 260 nm and 280 nm. The quality of the RNA was tested in 1% formaldehyde-agarose gel stained with ethidium bromide (EtBr).

3.6. Microarray Hybridization

Gene expression profiles were assessed using Gene Expression Service with the Illumina MouseRef-8 v2 Expression BeadChip® with Ambion Labelling. This BeadChip targets approximately 25,600 well-annotated RefSeq transcripts, over 19,100 unique genes.

Data were analyzed by using the GenomeStudio™ Software (Illumina, San Diego, CA, USA) following the manufacturer's instruction. Significant modulated genes were defined as those with an absolute fold change of >2.0 and an adjusted p value of <0.05.

Differentially expressed genes were classified according to their role(s) in cellular or metabolic pathways using the online GeneCodis analysis software for modular and singular enrichment analysis [39]. Gene Ontology (GO) analysis was also performed to describe the associated biological process of the differentially expressed genes overall [40].

3.7. Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR of selected genes was performed to validate microarray results of adipose samples by using RT^2 profiler PCR array (SABiosciences, Qiagen). The array was customized in 384 wells plates to contain a panel of genes specifically relevant to MAPK pathway, five different housekeeping genes and controls for genomic DNA contamination, reverse transcription and positive PCR controls. Dissociation curves were assessed to ensure the presence of a single amplicon. Reactions were performed with 50-fold diluted cDNA (1 ng/μL), 5 μL of USB VeryQuest SYBR Green qPCR Master Mix (2X) (Affymetrix, Madrid, Spain) and RNAse-free water being added to a final volume of 10 μL. Real-Time RT-PCR reactions were performed in 384-well plates and gene expression was determined using the 7900HT Real-Time PCR System (Life Technologies, Alcobendas, Spain). Cycling conditions

were initial activation step at 95 °C for 15 min; 3-steps cycling for 40 cycle including denaturation at 94 °C for 15 s, annealing at 58 °C for 30 s and extension at 70 °C for 30 s; dissociation curve at 95 °C for 15 s, 60 °C for 15 s, 95 °C for 15 s. Gene expression was quantified using the ΔΔCt method and fold-change values were reported as $2^{-(\Delta\Delta Ct)}$.

3.8. Statistical Analysis

Statistical analyses were carried out using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Apart from gene expression, data independent samples t-test was used when the corresponding assumptions were met; otherwise the non-parametrical Wilcoxon Mann-Whitney test was employed. $p < 0.05$ was considered significant. Results are presented as means ± SD.

Supplementary Materials

Supplementary materials can be accessed at: http://www.mdpi.com/1420-3049/20/02/2310/s1.

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Author Contributions

EG Performed experiments including animal studies and wrote the paper; AD Performed experiments, including animal studies and gene expression, and wrote the paper; MCC Performed gene expression experiments; JT-C Performed analysis of gene expression; DG-C Performed plasma biochemical determination; FV Conceived the idea, supervised research, and wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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Sample Availability: Samples of the compound are available from the authors.

Supplementary Materials

Table S1. Chemical composition of the Soyselect batch used in this study.

Energy Value	58 (247)	kcal/100 g (kJ/100 g)
Proteins (as sum of aminoacids after hydrolysis)	1.93	g/100 g
Total Fat	1.4	g/100 g
• Saturated Fatty Acids	0.14	g/100 g
• Monounsaturated Fatty Acids	<0.05	g/100 g
• Polyunsaturated Fatty Acids	0.21	g/100 g
• trans-Fatty Acids	<0.05	g/100 g
Total Dietary Fiber	<0.5	g/100 g
Sugars Composition		
Fructose, anhydrous	2.10	g/100 g
Glucose, anhydrous	0.73	g/100 g
Lactose, anhydrous	<0.10	g/100 g
Saccharose (Sucrose), anhydrous	6.70	g/100 g
Maltose, anhydrous	<0.10	g/100 g
Total Sugars	9.53	g/100 g
Moisture	3.9	g/100 g
Ash	2.54	g/100 g
Sodium	37.0	mg/100 g
Calcium	2.82	mg/100 g
Iron	0.32	mg/100 g
Cholesterol	<0.10	mg/100 g
Vitamin A (retinol)	<0.01	mg/100 g
B-Carotene	<0.02	mg/100 g
Vitamin C (Ascorbic Acid, HPLC)	<2.5	mg/100 g

Table S2. Soy isoflavones mix induced genes.

DEFB37	DEFB21	EIF3K	PAPOLB	C730025P13RIK	BEX4
DEFB38	HEXB	PRPS1L1	ADAM5	STK22B	RASL2-9
PRM1	4921520G13RIK	ST6GALNAC2	1700021C14RIK	2700055A20RIK	LOC100044177
SPINT4	OTTMUSG00000016790	OXCT2B	LCN13	SQRDL	CYP4A12A
OTTMUSG00000016293	BHLHB2	BCAS2	MRPS18C	KPNB1	1190002A17RIK
DEFB23	NLRP14	GM128	KRT14	D430015B01RIK	1700093K21RIK
DEFB39	COX6C	SMARCD3	SEC61G	4921507P07RIK	SLC2A3
OAZ3	NTSR2	DEFB29	ODF4	CLK1	SLC30A6
SMCP	TCP11	D19ERTD721E	MAP1LC3B	TAX1BP1	BCKDHB
4931407G18RIK	HOXD4	1700025K23RIK	ODF3	CAGE1	CCNL1
NPY	COX8C	A330021E22RIK	SOCS7	HGFAC	IRGM
FABP9	ARL8A	1700010M22RIK	WDR20B	NPAL2	TWISTNB
9230002F21RIK	CRISP4	ACRV1	LOC100047749	1700010A17RIK	SLTM
SPAG11	CLDN11	AKTIP	GM1679	XLR4A	RAB5C
OTTMUSG00000015852	DDX25	STARD10	CCNO	BC048546	RNF133
SPINK2	DYNLT3	CCDC113	RASSF3	MRPL20	YWHAB
SPINK8	C130090K23RIK	ENPP2	CDH1	RPO1-1	1700067P10RIK
AY761185	CAMK2B	CCT6B	PRF1	1300007L22RIK	WBP4
TNP1	MYCBPAP	ZMAT1	1700026L06RIK	FRK	ZFP654
CUZD1	2410116G06RIK	PICALM	GRHL2	AGPAT3	ZBBX
SPINK11	SETX	COQ2	4921517D21RIK	RRN3	PABPC2
DEFB2	LOC100042777	GAPDHS	A530050D06RIK	CIZ1	PLP1
TSSK6	DDC8	KIF2B	ANKRD46	LOC100047200	RAD52
INDO	LOC677317	MNS1	SLC1A1	LOC100048020	EG385328
RPL3L	KCNC4	GSTT3	UBQLNL	4933402J07RIK	PLA2G4F
ADAM32	TCOF1	CPEB2	SPZ1	LIP1	DAPL1
WFDC15B	SLFNL1	LCN8	1700054O13RIK	HSP90B1	CFB
ODF1	KLHL10	1700020D05RIK	VDAC2	4922505E12RIK	1700001C02RIK
DEFB11	CCDC92	1700065I17RIK	4833420G17RIK	ERGIC2	TLE2

Table S2. *Cont.*

WFDC10	PRM3	SERF1	FYCO1	ELAVL1	2610101N10RIK
DEFB19	LRRC50	1700011H14RIK	ATP6V1A	UHRF1BP1L	1700019H03RIK
ALDH6A1	GRB7	4732415M23RIK	4933422H20RIK	RASGRF1	THAP4
SPINLW1	PTGDS	MOD1	EMG1	DYNLRB2	DEFB12
RSPH1	1810015C04RIK	CTNNB1	LLGL2	NDUFB9	IQCF4
BC051142	LOC100039532	1700029H14RIK	TMEM51	CGGBP1	TCTEX1D2
TCFAP2B	GSTT4	NOTCH1	ACTL7B	TRIM36	NIT1
LCN12	MFAP3L	4930412F15RIK	MED28	ARRDC2	1110002B05RIK
TULP2	LOC100048622	NT5C1B	TMCO5	ARHGEF4	1700026D08RIK
LDHC	HCFC1	CHCHD7	COL16A1	TNP2	HOXD3
BC030476	PTPRB	IDH2	CYB561	ADAM28	SETD1B
BC048679	GLDC	NXF1	HIST2H2BE	H3F3B	ISYNA1
PGK2	TUBA3B	BC038286	1700012L04RIK	KRT8	CC2D2A
LOC435023	SYTL4	CUL1	USP20	1700007K09RIK	M6PR
SPATA19	LYPD4	HADHB	SMAD1	ATP5A1	DEFB10
GSG1	SLC7A4	TMC5	4930526D03RIK	MBOAT1	ZPBP
PGAM2	DDIT4L	HILS1	PTGES3	ZDHHC4	OTUD5
ZNRF4	ATP13A4	1700094C09RIK	HSD17B10	HNRPAB	ZC3H15
UBQLN3	DEFB42	AQP9	1700057K13RIK	TBC1D8	2400003C14RIK
GYKL1	SPATA6	1700049L16RIK	TEKT1	BC020002	SPO11
SVS4	ACTL6A	PRDX6-RS1	CCT8	ATF4	CLIC3
SERPINA1F	LRRC57	BC049635	SMEK2	1110017D15RIK	SLC38A5
HDAC6	TDRD6	EG432867	4931406C07RIK	MAPBPIP	EG654465
CLGN	CST9	ENOPH1	EXOSC8	LRRC34	IL10RB
KCNK1	YBX2	ACTL7A	SKP1A	HUWE1	LOC100045098
TSGA8	BGLAP-RS1	BZW1	MDH1B	4930404H21RIK	5133400G04RIK
CLDN3	PFN3	EG433365	CUGBP2	GM614	SGPP1
LY6F	SMC5L1	CLCNKB	REC8	PAIP2	CAPZA3

Table S2. *Cont.*

DBIL5	P2RX2	NOL14	SATB1	HSD3B2	CYB5R1
SVS5	1700034E13RIK	ARID2	HOOK2	PURB	AKAP3
4922502D21RIK	AI314180	CLDN4	WDR23	CSTL1	MTMR12
EIF2S3Y	NUP210	LOC100048480	KCTD2	LRRC46	5830403L16RIK
TESK1	MLF1	ADCY8	OTTMUSG00000015859	GNPDA2	CRYBA4
PLS3	DEFB40	ZFP263	SNX1	NDFIP2	PRPS2
PDZK1	SPINK10	H2-AA	RPRM	GLB1	LAMA1
HOXB4	ESPN	TCTE3	STAG3	1700123L14RIK	PQLC1
LOC669168	CDH16	ODF2	DPEP3	TAF13	COX7A2
AP3B2	KRT18	C4BP	TRIP12	MGL1	DRBP1
RNASE9	SUSD4	LOC217341	GM1698	R3HDM2	4933405O20RIK
AKR1C19	SPACA3	SRP14	LYZL4	PBP2	PGM2
HBB-B1	ALDH1A1	EG665378	RGMA	CDC42EP3	TMEM176B
WFDC6B	GKAP1	1600016N20RIK	LOC100044779	1700018C11RIK	SLC44A4
ADAM3	ACTG2	DDX21	DDX3X	PRSS21	PTPRE
PACRG	DEFB30	1700080E11RIK	ACTR3	1700019D03RIK	SNF8
CYP17A1	ACSBG2	ISG20	ST13	ARL1	ANKRD10
SEC11C	HRASLS5	PGRMC1	HMGB4	4930451I11RIK	ZC3H6
NPC2	D11WSU47E	FHL5	AHCTF1	SPAG6	PIWIL1
DNAJC5B	TCP10A	CYP1B1	NDUFB10	ECSIT	CSPP1
DAZAP2	EMB	HSDL1	SPERT	PTPN1	IZUMO1
ACADSB	SELK	H1FNT	LYZL1	TTC29	D030013I16RIK
DEFB15	TESP1	QRICH2	PLAA	SERINC2	MGC107098
MRPL52	SVS7	TMED9	DCUN1D5	TECTA	RPS3A
RHBDL2	LTF	CRISP1	MARCH10	CCDC54	HSPA2
4930563D23RIK	HSPA1L	DNALI1	PRPS1	GALNTL5	AKAP4
WFDC13	S100A10	SPATA20	RAG1AP1	CATSPER3	1700030J22RIK

Table S2. *Cont.*

RBM35A	LOC100048703	OXCT2A	GOT2	DEFB25	AARD
LOC100047619	DEFB43	LCN5	ARMCX1	TXNDC8	HOXD8
POLR2G	ACTRT2	DEFB20	1700029J07RIK	TXNDC12	DEFB18
ZXDA	RNASE12	PROM2	TXNDC2	SLCO4A1	2900010M23RIK
YWHAZ	9230104L09RIK	LYZL6	PABPC1	TJP2	TRIM39
TUBA3A	DNAJC10	TEDDM1	4930503B20RIK	UPK1B	BC089491
ADAM7	LYAR	ENPP1	KCNH3	TPP2	SPHK1

Table S3. Soy isoflavones mix repressed genes.

LYZ2	C1QB	TMEM9	2410166I05RIK	THRSP	TAP1
NNAT	LAMC1	IGFBP6	AGTR1A	GTRGEO22	CRY2
GSN	ARSB	NID2	LOX	IL1RL1L	2010316F05RIK
APCDD1	ADRB3	DGAT2	HIST1H2BH	APOC3	ORC6L
SERPINA3C	2210021J22RIK	2010311D03RIK	REEP5	RGS16	HEPH
VIM	MFAP5	LOC100046393	LOC100044204	GEMIN7	C1QBP
TMEM45B	TMEM131	2310058J06RIK	DYNC1LI2	LEPREL2	EPB4.9
SSPN	GPX7	TMEM159	MRPL11	MAPK3	SH3KBP1
SVEP1	CIDEA	YIF1A	LOC654426	2600010E01RIK	ME2
AGPAT2	UGT1A10	MC2R	MCOLN1	NUMA1	BC031181
LUM	PTRF	CD248	CHCHD4	MRPS16	MCTS1
MAPK1	PI16	SLC19A1	MRPL3	LOC100045697	HLCS
HSPA8	PLEKHB2	HTATIP2	FRMD6	TSPAN7	PLD2
ATP5F1	LOC641240	CHPT1	SRPX	COL14A1	4631427C17RIK
GPR81	MKNK1	ZFP207	TNFRSF1A	PGAM1	MS4A6D
AKR1B7	APOA2	ACSM3	STX18	2610204L23RIK	MYCL1
G0S2	EMP3	GSTT1	IER3IP1	EG630499	MAEA

Table S3. *Cont.*

PXMP2	SPR	PPP2R1A	GBP2	VTN	RPS27A
CRTAP	TGFBR3	COX7A2L	GPX1	PCX	2510010F15RIK
H2-AB1	LPIN1	EMD	ATP6V0E2	TMEM100	ARHGDIB
HTRA1	MRPL9	H2-M3	MRPL53	CMTM7	MAPK1
GPR109A	SLC1A5	NAP1L1	PEG3	HTRA3	9030624J02RIK
FSTL1	ADH1	EAR4	MPP1	TCEAL8	LOC100048613
RARRES2	EMILIN2	LOC100047937	OSTF1	TGFBI	CHST12
COL15A1	ADFP	PRKCDBP	IFI35	CCDC80	TM6SF1
RNF4	GALM	BLCAP	CD83	RPL18	AU019823
CD14	MSN	IFITM2	GSTO1	RBPMS2	H3F3A
RNASET2	LOC100046650	LOC381629	HCLS1	1500032L24RIK	ZCCHC14
FFAR2	PSME2	GSTP2	OSTM1	SNRPB	CDKN2C
EG433923	NAPEPLD	GNB1	FKBP9	TRAPPC4	9330186A19RIK
SPARC	SNF1LK2	D430028G21RIK	FOXO1	JAZF1	MRPL4
ORM1	HP	LOC100048413	SRD5A3	PDDC1	A730042J05RIK
C1QC	GNG10	2010004A03RIK	SCP2	CRYAB	VKORC1
CXX1C	GFPT2	OXCT1	P2RX4	ANXA6	YKT6
H2AFZ	DHRS1	TMED10	G6PDX	ENTPD2	AMOTL2
ARL6IP5	FADS3	HNRPF	LOC100047012	LRG1	CPNE8
CYP4B1	LGALS3BP	PRKCB1	CSPG4	UQCRH	2700038C09RIK
SORBS1	MXRA8	ABCD2	SEPP1	SCOTIN	D830050J10RIK
TRF	HSD11B1	RAP2A	NID1	PRTN3	EMILIN1
NTRK2	ITGA7	FOLR2	COPS6	HIST1H2BF	1110031B06RIK
SAA3	IAH1	MOBK1B	GSTP1	CLPTM1	NOL5A
ACTN4	PHKG1	DOCK1	DPEP1	HRAS1	PER3
SYNGR2	NPR3	COQ9	1110002N22RIK	CD68	LDHA

Table S3. *Cont.*

S100A1	SLC2A4	TSC22D1	SDF2	NDUFB6	BC038156
SUCNR1	SUMO3	CAPNS1	MPDU1	CLTA	APOE
HEBP1	MYADM	B4GALT1	1190002H23RIK	GMPR	GRB10
LGMN	ATP5L	DAD1	CHIC2	PPP1R8	TINAGL
LY6C1	TPPP3	UBE2E3	PDK4	RAC2	BCKDK
CXCL1	EPS8	CXX1A	AHNAK	PLTP	2700078K21RIK
TIMP4	SOD3	RPS2	CXCL9	KCNJ8	CDK2AP2
LOC100045567	IDH1	CAR3	IARS2	LOC100047653	ACSF3
CEBPA	ITGA1	BMP3	WWTR1	MED11	PPAPDC1
PLA1A	CYC1	GPD1	RPS7	EG622320	SPCS1
CISH	PHGDH	NDUFS2	MTPN	FCGRT	A730008L03RIK
COL4A2	EMP1	THBS2	PODN	AP2A2	SNTG2
SERPING1	S100A8	MRAP	CYBA	PPAP2C	MTAP
TSPO	DARC	SCARA5	CHURC1	TMEM41A	ORAI3
PHLDA3	1190005I06RIK	PCBP1	RTN2	KDELR2	D930001I22RIK
RPS6	PRDX3	2310044H10RIK	PPCS	LRRC8	RPS4X
MMP2	MNAT1	OTTMUSG00000000971	OLFM1	AW146242	CCM2
PRKCB1	NDUFC2	TMEM9B	MGL2	DHRS7	CASP6
CTSF	2310008M10RIK	MRVI1	TMEM43	NFKB1	MAPK14
SLIT3	TSPAN3	NOMO1	CALU	HFE	IL2RG
H2-EB1	COL6A2	MRC1	EPB4.1L1	EPDR1	TBC1D9B
ENC1	PLIN	TGFBR2	FGFRL1	EAR2	TMEM16K
CD74	5730437N04RIK	LOC100047353	CIITA	VPS35	TPST1
EG667977	ALOX5AP	RAB18	PEPD	ITIH5	CAML
LEP	LOC100043257	SGTA	BC051227	IL1B	CTSH
EHD2	POLD4	RBCK1	L7RN6	TIMP1	ROCK1

Table S3. *Cont.*

EEF2	BC004044	SLC11A2	MGC41689	COPZ1	PPP5C
SORL1	VNN3	ERRFI1	THRA	PTGES	SCAMP3
TRP53INP2	C1QA	BC013712	MAN2A1	MGST1	HRAS1
4833421E05RIK	9030224M15RIK	TNNC2	ACAA2	MRPL34	

Table S4. Validated genes by qRT-PCR.

Upregulated Genes		**Downregulated Genes**
DEFB37	CLDN3	VIM
DEFB38	PDZK1	LUM
PRM1	ADAM3	MAPK1
SPINT4	SEC11C	ATP5F1
DEFB39	DEFB15	FSTL1
OAZ3	MRPL52	RARRES2
SMCP	POLR2G	CD14
NPY	YWHAZ	PRKCB1
FABP9	DEFB21	GPX7
SPINK2	COX6C	MKNK1
SPINK8	TCP11	HSD11B1
TNP1	COX8C	SOD3
CUZD1	CRISP4	PRKCB1
SPINK11	CLDN11	LOX
DEFB2	SLFNL1	TNFRSF1A
TSSK6	LRRC50	FOXO1
RPL3L	GRB7	CYBA
WFDC15B	PTGDS	TGFBI
DEFB11	ATP13A4	HRAS1
WFDC10	DEFB40	NFKB1
SPINLW1	SPINK10	MAPK1

Table S4. *Cont.*

Upregulated Genes		Downregulated Genes
LDHC	CDH16	MAPK14
PGK2	KRT18	ROCK1
SPATA19	DEFB30	PPP5C
GSG1	HRASLS5	HRAS1
ZNRF4	LTF	
SERPINA1F	EIF3K	
CLGN	CYP1B1	
KCNK1	PTPN1	
TSGA8	SPHK1	

Chapter 2. *Hydroxytyrosol*

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One-week administration of hydroxytyrosol to humans does not activate Phase II enzymes

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ABSTRACT

The notion that (poly)phenols act as direct free radical scavengers is being challenged by mere chemical and biochemical considerations such as bioavailability and intracellular concentrations. An alternative hypothesis that is gaining considerable traction is that (poly)phenols are processed by the body as xenobiotics via the Keap1/Nrf2/ARE signaling axis, leading to the induction of Phase II enzymes. However, there are no solid human data to confirm this interesting supposition. In this study, we tested the activities of hydroxytyrosol (HT) on Phase II enzymes' expression in a double-blind, randomized, placebo-controlled study. We tested two HT doses, i.e. 5 and 25 mg/d, vs. placebo following a Latin square design. We report that HT is well tolerated but does not significantly modify Phase II enzyme expression in peripheral blood mononuclear cells. Moreover, we were unable to record significant effects on a variety of surrogate markers of cardiovascular disease such as lipid profile and inflammation and oxidation markers. Available evidence indicates that the "hormesis hypothesis" that (poly)phenols activate Phase II enzymes requires solid human confirmation that might be provided by future trials.

This study is registered at ClinicalTrials.gov (identifier: NCT02273622).

1. Introduction

(Poly)phenols are the products of plants' secondary metabolism and are endowed with several botanical activities [1]. Notable examples include roles in pollination, color, insect repelling, and cellular signal transduction. In addition, since the Zutphen Study [2] (poly)phenol consumption by humans is being consistently associated with better cardiovascular prognosis and chemoprevention. Pharma-nutritionists are trying to explain the molecular mechanisms responsible for the purported healthful activities of (poly)phenols; major emphasis is being placed on their antioxidant actions, which would counteract the noxious effects of reactive oxygen species and free radicals. However, the widespread notion that (poly)phenols act as direct free radical scavengers is challenged by mere chemical and biochemical considerations such as bioavailability and intracellular concentrations (namely, as compared with endogenous antioxidants), reaction kinetics, etc. [3].

An alternative hypothesis that is gaining considerable traction is that (poly)phenols are processed by the body as xenobiotics [3,4]. Therefore, they stimulate stress-related cell signaling pathways that result in increased expression of genes encoding cytoprotective genes. In particular, Nrf2 (NF-E2-related factor 2) is a transcription factor which binds to the Antioxidant Response Element (ARE) in cells and thus regulates enzymes involved in antioxidant functions or detoxification such as thioredoxin reductase-1 and glutathione peroxidases [3]. According to the hormesis theory, (poly)phenols paradoxically act on the Keap1/Nrf2/ARE signaling axis to produce additive increases in electrophilic signaling that results in the induction of Phase II enzymes and increased nucleophilic substrates, such as glutathione, thioredoxin, and NADPH. In brief, (poly)phenols likely exert indirect rather than direct antioxidant actions. However, there are no solid human data to confirm this interesting supposition.

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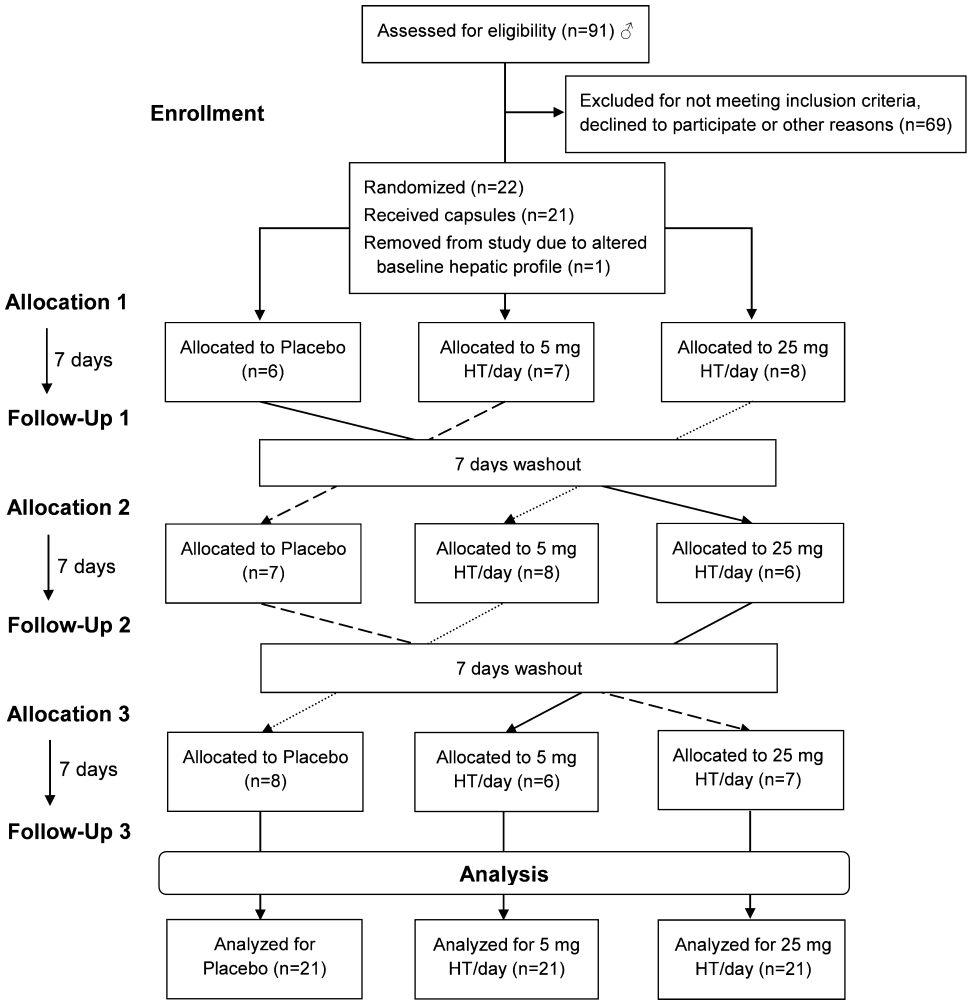

Fig. 1. Study flowchart.

In this study, we tested the activities of hydroxytyrosol (HT) on Phase II enzymes' expression in a double-blind, randomized, placebo-controlled study.

2. Materials and methods

2.1. Subjects and study design

The study protocol was approved by the local Ethics committee and was fully explained to the participants. Written informed consent was obtained by all subjects prior to starting the trial. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and is registered at ClinicalTrials.gov (identifier: NCT02273622).

This was a double-blind, randomized, placebo-controlled study. We tested two HT doses, i.e. 5 and 25 mg/d provided via administration of Hytolive® (Genosa, Madrid, Spain), an olive mill waste water (OMWW) extract selectively enriched in HT, i.e. devoid of oleuropein or other HT-containing secoiridoids as assessed by HPLC [5], via anion-exchange chromatography. Twenty-two apparently healthy volunteers were recruited from within the IMDEA-Food Genyal platform database. Inclusion criteria were: age between 20 and 40 years; adequate understanding of the study; willingness to complete the entire treatment. Exclusion criteria were: body mass index <19 or >26; diagnosis of diabetes mellitus, hypertension, dyslipidemia or other cardiometabolic disorders; impaired cognitive function; diagnosed hepatic, renal, or cardiovascular disease; allergy to olives and their derivatives; pharmacological therapies; and habitual smoking. To our knowledge, there are no previous studies testing the activities of HT on Phase II enzymes' expression in humans. Therefore, we designed a pilot study and we could not calculate power. However, the use of repeated measures increases the power to detect treatment differences in mean levels of the outcome measure over time.

We followed a Latin square design: after one-week washout, i.e. olive-free diet, subjects were randomly assigned to either the placebo (maltodextrin), 5 mg/d HT, or 25 mg/d HT group (Fig. 1). The complete capsule composition is described in Supplementary Table 1. Administration of each treatment was carried out for one week, followed by a one-week washout after which treatments were switched. Volunteers were instructed not to consume any olive-based products or medication throughout the study and were instructed to write down any occurrences (consumption of prohibited foods, medication intake, etc.) and intolerance issues (diarrhea, acidity, nausea, abdominal distension, or halitosis) in questionnaires. Capsules were provided in bottles labeled A, B, or C and their contents were unknown to both the volunteers and the nurse who administered the bottles. Compliance was assessed by capsule count.

Blood samples were drawn and anthropometric data (height, weight, body mass index (BMI), bioelectric impedance analysis (BIA)) and vital constants (systolic (SBP) and diastolic blood pressure (DBP), and heart rate) were monitored at each visit. Morning urine was also collected at each time point.

2.2. Biochemical analyses

Triacylglycerol (TG), total (TC), low-density (LDL-c), and high-density (HDL-c) cholesterol concentrations were measured by routine laboratory (Laboratorio CQS, Madrid, Spain, which follows the UNE-ISO 15189:2007 directives) methods. Urea, creatinine, hepatic enzymes: glutamyl oxaloacetic transaminase (GOT), glutamic-pyruvatetransaminase (GPT) and gamma glutamyltransferase (GGT), bilirubin, and alkaline phosphatase (AP) were also measured by routine laboratory methods. The concentration of oxidized LDL was measured by sandwich enzyme-linked immunosorbent assay (ELISA) by using the monoclonal antibody mAb-4E6 (Mercodia AB, Sweden).

Urinary thromboxane B_2 and total isoprostanes were quantified by competitive ELISA (Enzo Biochem, Inc., NY, USA and Oxford Biomedical Research, MI, USA, respectively).

2.3. Multiplex bead immunoassay

In a subset of samples, plasma concentrations of Interleukin (IL)-6, IL-8, IL-10, IL-17, monocyte chemoattractant protein 1 (MCP-1); Tumor necrosis factor alpha (TNFα), and vascular endothelial growth factor (VEGF) were measured using a magnetic bead-based immunoassay (MAGPIX-Luminex) kit from Millipore, following the manufacturer's instructions. A minimum of 50 beads per parameter were analyzed by the MAGPIX-Luminex system. Raw data (median fluorescence intensity, MFI) were analyzed with the xPONENT software 4.1.

2.4. Isolation of peripheral blood mononuclear cells (PBMCs)

Subjects were instructed to fast overnight before each blood collection. Blood samples were collected between 8 and 10 AM to minimize circadian variations. Blood samples were collected in heparinized tubes (BD Vacutainer, Franklin Lakes, NJ, USA) at each visit, processed within 2 h after extraction and used to PBMCs. Isolation was carried out under sterile conditions to avoid monocytes activation. Whole blood was diluted (1:1) with phosphate buffer solution (PBS) and centrifuged by density gradient with Histopaque-1077 (Sigma–Aldrich, Madrid, Spain) according to the manufacturer's instructions. After collection, PBMCs were washed twice with PBS, homogenized in Qiazol (Qiagen, Madrid, Spain) and stored at −80 °C prior to RNA extraction.

2.5. RNA extraction and microarray samples preparation

Total RNA was extracted and purified from homogenized PBMCs with miRNeasy minikit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Recovered RNA's concentration and integrity were verified using a Nanodrop ND-1000 spectrophotometer (Nanodrop TechnologyR, Cambridge, UK) and an Agilent 2100 Bioanalyzer (Agilent, Madrid, Spain).

2.6. Gene expression analysis

After DNAse I treatment (Invitrogen, Madrid, Spain), reverse transcription was performed with miScript® II Reverse Transcription kit (Qiagen, Germantown, MD) according to the manufacturer's guidelines. RT-qPCR reactions were performed in 384-well plates and gene expression was determined using the 7900HT Real-Time PCR System (Life Technologies, Spain). Reactions were performed with 5 μL of miScript SYBR® Green qPCR Master Mix (Qiagen, Madrid, Spain). Cycling conditions were 15 min at 95 °C for one cycle, then 40 cycles at 94 °C for 15 s, 58 °C for 30 s, 70 °C for 30 s, for each gene. The dissociation stage was analyzed at 95 °C for 15 s, followed by one cycle at 60 °C for 15 s, and 95 °C for 15 s. Gene expression analysis was carried out for twenty Phase II enzymes (primers can be found in Supplementary Table 2). Three potential reference genes–GAPDH, HPLPO, and ACTB–were tested in order to select the most stable to be used in target gene's normalization, which according to Normfinder was the latter. Reactions were run in triplicate and relative expression of Phase II enzymes was calculated by the comparative Ct method and presented as $2^{-\Delta\Delta Ct}$.

2.7. Statistical analyses

Continuous descriptive variables were expressed as mean ± SEM. Two-way repeated measures ANOVA was used to evaluate the effects of time (t_1 and t_2), treatment (A, B, C) and the time × treatment interaction. A Bonferroni correction for multiple analyses was applied and models were adjusted for age and sequence (ABC/CAB/BCA) as covariates. All statistical analyses were considered as bilateral and significance was set at $p < 0.05$. Data were analyzed with R Statistical Software version 3.1.1 (www.r-project.org).

3. Results

One volunteer was excluded from the study because he exhibited abnormal basal bilirubin concentrations (Fig. 1); attrition rate was, therefore, zero. Both 5 and 25 mg HT doses were well-tolerated and no adverse effect was reported.

No differences in anthropometric variables, such as weight or body mass index, were recorded and no significant variations in vital signs such as blood pressure were noted either (Table 1).

Table 1
Anthropometric parameters and vital signs.

Measured parameters	A		B		C		Effect (p)*		
	Initial	Final	Initial	Final	Initial	Final	Time	Treatment	Time × treatment
Weight (kg)	73.31 (1.58)	73.1 (1.61)	73.1 (1.58)	72.85 (1.57)	73.07 (1.62)	72.73 (1.64)	0.002*	0.989	0.833
B.M.I (kg/m^2)	23.69 (0.41)	23.62 (0.42)	23.62 (0.43)	23.55 (0.43)	23.61 (0.44)	23.49 (0.45)	0.002*	0.984	0.721
FM (%)	19.99 (1.16)	20.01 (1.23)	20.1 (1.23)	20.02 (1.24)	19.87 (1.3)	19.82 (1.25)	0.787	0.991	0.944
MM (%)	39.81 (0.71)	39.72 (0.77)	39.69 (0.77)	39.67 (0.77)	39.78 (0.81)	39.82 (0.78)	0.805	0.993	0.821
AVF	5.9 (0.42)	5.95 (0.43)	5.86 (0.44)	5.76 (0.44)	5.86 (0.44)	5.76 (0.45)	0.263	0.973	0.287
SBP (mmHg)	130 (2.35)	131.1 (1.98)	129.62 (2.84)	131.19 (2.18)	130.14 (2.47)	128.71 (2.17)	0.681	0.920	0.424
DBP (mmHg)	70.1 (1.51)	72.48 (1.48)	71.57 (1.61)	71.71 (1.46)	70.29 (1.21)	71.24 (1.76)	0.1622	0.878	0.531
HR (Beat/min)	60.19 (3.96)	69 (2.91)	64.05 (2.15)	67.76 (2.5)	63.38 (2.01)	66.1 (2.75)	0.0013*	0.893	0.214

A: 250 mg Hytolive (25 mg hydroxytyrosol); B: 50 mg Hytolive (5 mg hydroxytyrosol); C: Placebo. B.M.I, body mass index; FM, Fat mass; MM: Muscle mass measured by BIA: bioelectrical impedance analysis; AVF, abdominal visceral fat (measured by BIA); SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, Heart Rate. Effect (p); Time: indicates the evolution in the same group from the beginning to the end of the intervention. Treatment: indicates differences between treatments (A, B, C) independently of time. Time × treatment: indicates the differences in evolution between groups as a result of treatment received (A, B, C). The p value for interaction was adjusted for age and sequence of consumption as covariates. Data are means (SEM).

* $p < 0.05$.

Table 2
Biochemical parameters evaluated in each of the visits.

Measured parameters	A		B		C		Effect (p)*		
	Initial	Final	Initial	Final	Initial	Final	Time	Treatment	Time × treatment
TC (mg/dl)	169.3 (6.54)	169.7 (6.51)	168.6 (6.39)	173.9 (6.8)	170.8 (6.77)	169.4 (5.97)	0.388	0.975	0.258
HDL (mg/dl)	52.83 (2.57)	51.04 (2.06)	52.17 (2.66)	52.14 (2.44)	51.84 (2.26)	50.87 (2.48)	0.229	0.960	0.644
LDL (mg/dl)	99.47 (5.47)	103.0 (5.41)	101.3 (5.47)	106.2 (5.7)	102.5 (5.78)	103.7 (5.17)	0.031*	0.923	0.559
TG (mg/dl)	85.1 (7.26)	78.24 (6.15)	75.81 (4.2)	77.6 (6.57)	82 (8.05)	74.6 (4.96)	0.163	0.793	0.371
hs-CRP (mg/dl)	0.07 (0.02)	0.16 (0.06)	0.09 (0.02)	0.05 (0.01)	0.09 (0.02)	0.05 (0.01)	0.47	0.117	0.049*
TXB_2 (ng/ml)	1.55 (0.2)	1.71 (0.14)	1.97 (0.26)	1.72 (0.16)	2.17 (0.18)	1.83 (0.3)	0.253	0.316	0.222
TXB_2/Creat (μg/g)	0.87 (0.06)	0.86 (0.05)	1.03 (0.10)	0.9 (0.06)	1.01 (0.06)	0.94 (0.08)	0.097	0.373	0.502
LDL-ox (U/l)	42.6 (2.32)	41.34 (2.04)	42.19 (2.31)	43.95 (2.56)	41.28 (2.84)	41.34 (2.31)	0.849	0.814	0.445
Isop (ng/ml)	2.99 (0.34)	3.59 (0.41)	3.71 (0.41)	3.78 (0.42)	4.78 (0.63)	3.38 (0.56)	0.477	0.298	0.052*
Isop/Creat (μg/g)	1.64 (0.08)	1.75 (0.1)	1.92 (0.13)	2.05 (0.17)	2.42 (0.33)	1.74 (0.14)	0.257	0.128	0.020*
GOT/AAT (UI/l)	19.71 (1.26)	19.95 (1.18)	18.05 (0.79)	18.9 (0.96)	19.52 (0.77)	19.33 (1.09)	0.525	0.520	0.662
GPT/AT (UI/l)	19.33 (1.9)	19.67 (1.79)	18.38 (1.49)	18.24 (1.38)	20.0 (1.6)	18.62 (1.17)	0.458	0.744	0.403
GGT (UI/l)	21.07 (1.99)	21.48 (2.44)	22.38 (2.27)	21.89 (2.0)	21.69 (2.07)	20.66 (1.78)	0.260	0.909	0.196
TB (mg/dl)	1.1 (0.08)	1.16 (0.1)	1.16 (0.11)	1.12 (0.09)	1.06 (0.08)	1.21 (0.1)	0.148	0.994	0.143
AP (UI/l)	55.95 (2.92)	64.33 (3.89)	58.86 (3.31)	62.86 (3.23)	57.76 (3.6)	61.76 (3.3)	0.001*	0.967	0.204
Urine creatinin (mg/dl)	177.5 (16.9)	202.2 (15.9)	190.2 (14.5)	193.6 (14.0)	215.7 (14.8)	184.8 (14.1)	0.910	0.814	0.024*

A: 250 mg Hytolive (25 mg hydroxytyrosol); B: 50 mg Hytolive (5 mg hydroxytyrosol); C: Placebo. TC: total cholesterol; HDL: high-density lipoprotein; LDL: low-density lipoprotein; TG:Triglycerides; hs-CRP: high-sensitive C-reactive protein; TXB2: Thromboxane B2; TXB2/Creat: TXB2 ratio and creatinine in urine; LDL-ox: Oxidized Low Density Lipoprotein; Isop: Isoprostanes, Isop/Creat: Isoprostanes ratio and creatinine in urine; GOT: glutamyl oxaloacetic transaminase; GPT: glutamic-pyruvatetransaminase; GGT: Gamma Glutamyltransferase; TB: total bilirubin; AP: alkaline phosphatase. Time: indicates the evolution in each group from beginning to end of the intervention. Treatment: differences between treatments (A, B, C) independently of time. Time × treatment: differences in evolution between groups as a result of treatment received (A, B, C). Age and sequence of consumption were used as covariates. Data are means (SEM).

* $p < 0.05$.

Table 3
Cytokine levels after the consumption of the study products (A and C).

Measured parameters	A		C		Effect (p)*		
	Initial	Final	Initial	Final	Time	Treatment	Time × treatment
IL-10 (pg/ml)	2.22 (0.97)	1.36 (0.41)	0.79 (0.16)	1.71 (0.97)	0.96	0.46	0.17
IL-17 (pg/ml)	30.7 (10.2)	29.2 (8.18)	14.3 (2.61)	15.3 (3.98)	0.91	0.12	0.58
IL-6 (pg/ml)	10.6 (4.99)	11.5 (5.73)	6.20 (2.80)	5.20 (1.51)	0.96	0.18	0.45
IL-8 (pg/ml)	30.4 (9.57)	28.1 (8.56)	14.3 (4.43)	21.4 (9.92)	0.50	0.31	0.20
MCP-1 (pg/ml)	486 (54.0)	472 (54.1)	412 (37.0)	446 (43.5)	0.52	0.36	0.14
TNF-α (pg/ml)	8.99 (1.02)	8.31 (0.88)	7.37 (0.64)	6.74 (0.54)	0.04*	0.12	0.92
VEGF (pg/ml)	605 (164)	682 (182)	577 (110)	497 (98.2)	0.98	0.53	0.10

A: 250 mg Hytolive (25 mg hydroxytyrosol); C: Placebo. $n = 10$ in both groups, randomly selected. IL-10: Interleukin 10; IL-17: Interleukin 17; IL-6: Interleukin 6; IL-8: Interleukin 8; MCP-1: Monocyte chemoattractant protein 1; TNF-α: Tumor necrosis factor alpha; VEGF: Vascular endothelial growth factor. Data are means (SEM).

* $p < 0.05$.

Table 2 reports the plasma concentrations of selected surrogate markers of cardiovascular disease and hepatic enzymes. We only recorded significant time × treatment interactions for isoprostanes (markers of oxidation) and hs-CRP (marker of inflammation). Of note, hepatic function enzymes, which are – alas – rarely measured in nutraceutical studies, were not modified by either treatment.

In a subset of samples, namely those who received the highest HT dose vs. controls, we also quantified an array of plasma cytokines (Table 3) and did not find any significant variation induced by HT.

Phase II enzyme expression in PBMCs is reported in Table 4. We only documented a time effect on GSTO1 and GSTP1 levels, whereas all other genes were unaffected by the provision of HT.

4. Discussion

The notion that (poly)phenols trigger stress signaling mediated by Nrf-2 and Phase II enzymes is, nowadays, predominant over that of their plain antioxidant actions [3,6]. However, this hypothesis has never been confirmed in humans (to the best of our knowledge), with the exception of Hofmann et al. [7], who reported modest yet significant increases of glutathione *S*-transferase P1 (hGSTP1) protein expression in leukocytes of healthy volunteers given fruit juice. In brief, while biochemical verification of Nrf-2 mediated hormesis is strong, human evidence is scant or non-existent.

In this paper we report that HT, administered at two different doses in a Latin-square design study was well tolerated (as shown by the unaltered levels of hepatic enzymes, Table 2), but does not significantly modify Phase II enzyme expression in PBMCs. Moreover, we were unable to record significant effects on a variety of surrogate markers of cardiovascular disease such as lipid profile and inflammation and oxidation markers. Actually, we computed a non-significant increase in plasma hs-CRP and in urinary isoprostanes concentrations. The latter finding is in agreement with that of Leger el al. [8] and in disagreement with Visioli at al [9], who, however, administered extra virgin olive oils rather than HT. Because these differences were non-significant it is difficult to comment on their true physiological relevance; however, these data reiterate the concept that (poly)phenols and other minor food components exert biological effects whose nature and extent depend on the matrix, i.e. as part of food or provided as pharma-nutritional formulations.

We chose hydroxytyrosol (the foremost component of extra virgin olive oil [10]) as paradigmatic example of (poly)phenols because (1) it is the only phenolic molecule that received a European Food Safety Authority (EFSA) health claim [11]; (2) it has been shown to increase Nrf-2 expression in vitro [12,13] albeit at non-physiological doses [14]; (3) a "xeno-hormesis" postulation has also been proposed to explain the salubrious actions of extra virgin olive oil phenolics [15]; and (4) a previous human study with an olive mill waste water (OMWW) preparation reported increased glutathione levels short-term in human volunteers [16]. A glutathione effect was also recorded in rats [17] and a short-term

Table 4
Gene expression of Phase II enzymes after the consumption of the study products (A, B and C).

Phase II enzymes	Initial	Final						Effect (p)*		
		A		B		C		Time	Treatment	Time × treatment
NQO1	1	1.11	(0.11)	0.81	(0.07)	1.50	(0.61)	0.79	0.58	0.35
NQO2	1	1.79	(0.87)	1.04	(0.08)	1.05	(0.09)	0.74	0.66	0.95
GSTA1	1	1.28	(0.32)	1.32	(0.24)	1.01	(0.17)	0.38	0.93	0.45
GSTA4	1	1.01	(0.10)	1.11	(0.09)	1.10	(0.13)	0.73	0.79	0.58
GSTK1	1	1.27	(0.35)	0.99	(0.07)	1.03	(0.12)	0.87	0.42	0.96
GSTM1	1	1.22	(0.13)	0.89	(0.06)	1.19	(0.24)	0.40	0.89	0.16
GSTM2	1	1.73	(0.37)	1.20	(0.15)	1.54	(0.44)	0.12	0.92	0.97
GSTM3	1	1.16	(0.17)	1.15	(0.12)	1.06	(0.15)	0.96	0.92	0.46
GSTM4	1	1.22	(0.17)	0.97	(0.07)	1.19	(0.29)	0.63	0.89	0.29
GSTM5	1	1.39	(0.39)	1.07	(0.15)	1.18	(0.24)	0.36	0.88	0.95
GSTO1	1	0.90	(0.09)	1.18	(0.15)	0.90	(0.08)	0.03*	0.65	0.25
GSTO2	1	0.97	(0.08)	0.93	(0.08)	1.11	(0.11)	0.30	0.82	0.69
GSTP1	1	1.24	(0.09)	1.28	(0.16)	1.46	(0.25)	0.02*	0.85	0.99
GSTT1	1	0.94	(0.07)	1.06	(0.09)	1.22	(0.21)	0.73	0.84	0.56
GSTT2	1	1.58	(0.26)	1.22	(0.15)	1.08	(0.14)	0.50	0.59	0.29
HNMT	1	0.97	(0.08)	1.02	(0.10)	1.02	(0.08)	0.56	0.94	0.63
INMT	1	1.00	(0.13)	1.13	(0.15)	1.03	(0.15)	0.09	0.54	0.52
MGST1	1	1.06	(0.13)	1.19	(0.13)	1.05	(0.10)	0.98	0.70	0.22
MGST2	1	1.01	(0.07)	1.02	(0.08)	1.06	(0.13)	0.31	0.89	0.55
MGST3	1	0.90	(0.08)	1.13	(0.08)	1.05	(0.11)	0.16	0.92	0.17

A: 250 mg Hytolive (25 mg hydroxytyrosol); B: 50 mg Hytolive (5 mg hydroxytyrosol); C: Placebo. NQO1: NADPH dehydrogenase, quinone 1; NQO2: NADPH dehydrogenase, quinone 2; GSTA1: Glutathione S-transferase alpha 1; GSTA4: Glutathione S-transferase alpha 4; GSTK1: Glutathione S-transferase kappa 1; GSTM1: Glutathione S-transferase mu 1; GSTM2: Glutathione S-transferase mu 2; GSTM3: Glutathione S-transferase mu 3; GSTM4: Glutathione S-transferase mu 4; GSTM5: Glutathione S-transferase mu 5; GSTO1: Glutathione S-transferase omega 1; GSTO2: Glutathione S-transferase omega 2; GSTP1: Glutathione S-transferase pi 1; GSTT1: Glutathione S-transferase theta 1; GSTT2: Glutathione S-transferase theta 2; HNMT: Histamine N-methyltransferase; INMT: Indolethylamine N-methyltransferase; MGST1: Microsomal glutathione S-transferase 1; MGST2: Microsomal glutathione S-transferase 2; MGST3: Microsomal glutathione S-transferase 3. Data are fold change means (SEM).

* $p < 0.05$.

increase in skeletal muscle glutathione concentrations after exercise was reported by Bast and Haenen, who administered an HT-rich olive extract (providing 200 mg of HT) to human volunteers [18]. Finally, a nutrigenomic study carried out in mice reported that HT – in nutritionally relevant amounts – is able to positively modulate the glutathione-driven antioxidant enzymatic machinery in adipose tissue [19].

Of note, Visioli et al. [16] previously suggested that part of the purported healthful activities of HT might be governed by the antioxidant response element (ARE)-mediated increase in Phase II enzyme expression. Therefore, we undertook the current study to verify this premise in a controlled setting. Indeed, we also wanted to verify in humans the now popular notion that describes (poly)phenols as activators of the stress response pathways. We used PMBCs because they are easily accessible, express many genes previously believed to be restricted to non-blood tissues responding to macro- or micro-environment alterations in organs, and have been shown to be suitable for nutritional studies, reflecting specific effects of diets or nutrients [20–22].

To date, there are two human studies that have been performed with OMWW and one where the authors administered pure HT [8,23,24]. The former reported anti-thrombogenicity [8,23], while the latter only addressed absorption and disposition and did not inform on biochemical data [24].

Several hypotheses can be formulated to explain the discrepancy between our current results and those obtained with high-polyphenol extra virgin olive oil or those of Visioli et al. [16], namely in terms of oxLDL [25] and platelet aggregation [8,23] or oxidation and inflammatory markers [9]. First and foremost, we administered two doses of HT, i.e. 5 and 25 mg/d for one week. Higher doses might produce the hypothesized effects and are supposed to be safe. However, it is noteworthy that the EFSA health-claim [11] relates to 5 mg/d of HT and HT-containing phenols as protectors of LDL from oxidation. From a practical viewpoint this implies that formulations that provide HT amounts higher than this dose might have to deal with some regulatory issues, even though the safety profile of HT is excellent [26–28] as confirmed by the current study (Table 2). As a matter of fact, Visioli and Galli administered 50 mg of HT and recorded effects on TXB_2 production by serum short term, i.e. 1 h after intake [23]. These data were confirmed by Leger et al. [8], who provided five type I diabetic patients with 25 mg of HT the first day and 12.5 mg/day the following 3 days. A 46% decrease in the serum TXB_2 production after blood clotting at T (4d) was recorded [8]. Whether high doses of (poly)phenols trigger other short-term effects such as Phase II enzyme expression remains to be verified in humans. Also, the activation of Phase II enzymes might be organ-specific [29]; whether sustained hepatic Phase II enzymes activation occurs in humans would be very difficult to ascertain for obvious ethical and practical reasons.

Another important issue that the nutraceutical world is trying to address is that of healthy volunteers vs. patients [30]. Clinical trials of nutraceuticals, supplements, or functional foods in patients clash with obvious ethical guidelines; as a result, these preparations are often added to established, top-quality therapy, therefore making it difficult to ascertain their real contribution. One notable example is that of omega 3 fatty acids [31,32]. Our cohort of healthy volunteers exhibited normal-range concentrations of surrogate markers of cardiovascular disease at baseline: further reduction of such concentrations might be difficult to achieve. Conversely, HT might speculatively be beneficially used as medical food [33], i.e. as adjunct therapy of cardiovascular and other inflammation-based diseases [34] or to metabolic syndrome patients, pending confirmation by appropriate clinical trials [35].

Finally, it is worth reiterating that nutraceuticals and functional foods must exert modest (and often undetectable based on current methodologies) physiological effects which, however, could produce remarkable health activities if taking place long-term [36]. Indeed, epidemiological evidence of healthful activities is strong and corroborates the “sub-clinical, lifetime exposure” proposition.

In conclusion, the physiological activities of HT are – as of today – limited to inhibition of TXB_2 production by serum, leading to a possible prevention of thrombotic and micro-thrombotic processes. Most important, the “hormesis hypothesis” that (poly)phenols

activate Phase II enzymes requires solid human confirmation that might be provided by future trials.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2015.03.018.

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Supplementary Table 1. Composition of the capsules used in the study.

Ingredient	A	B	C
	Weight (mg)		
OMWW extract >10% hydroxytyrosol	250	50	-
Maltodextrin (maltosweet 180)	-	-	253,44
Microcrystalline cellulose type 101-CG	3,44	203,44	-
Precipitated silica, IBERSIL® d250 (46,7%si)-CG	2,64	2,64	2,64
Magnesium stearate-CG	7,92	7,92	7,92
Caps"1"bl	76	76	76
Total weight	340	340	340

OMWW, olive mill waste water.

Supplementary Table 2. Primer sequences used and expected amplicon sizes.

Gene symbol	Gene name	Primer sequence (5´-3´)	Amplicon size (bp)
NQO1	NAD(P)H dehydrogenase, quinone 1	Fw:GAAGAGCACTGATCGTACTGGC Rev:GGATACTGAAAGTTCGCAGGG	196
NQO2	NAD(P)H dehydrogenase, quinone 2	Fw:CCACGAAGCCTACAAGCAAAG Rev:CCAGTACAGCGGGAACTGAAATA	103
GSTA1	Glutathione S-transferase alpha 1	Fw:CTGCCCGTATGTCCACCTG Rev:AGCTCCTCGACGTAGTAGAGA	185
GSTA4	Glutathione S-transferase alpha 4	Fw:CCGGATGGAGTCCGTGAGAT Rev: GGGCACTTGTTGGAACAGC	127
GSTK1	Glutathione S-transferase kappa 1	Fw:TCTGGAAAAGATCGCAACGC Rev:GCCCAAAGGCTCCGTATCTG	83
GSTM1	Glutathione S-transferase mu 1	Fw:TCTGCCCTACTTGATTGATGGG Rev:TCCACACGAATCTTCTCCTCT	117
GSTM2	Glutathione S-transferase mu 2	Fw:TGTGCGGGGAATCAGAAAAGG Rev:CTGGGTCATAGCAGAGTTTGG	99
GSTM3	Glutathione S-transferase mu 3	Fw:TCGTGCGAGTCGTCTATGGT Rev:TCTCCTCATAAGAGGTATCCGTG	100
GSTM4	Glutathione S-transferase mu 4	Fw:TCTGCCCTACTTGATTGATGGG Rev:TCCACACGAATCTTCTCCTCT	117
GSTM5	Glutathione S-transferase mu 5	Fw:CCATCCTGCGCTACATTGC Rev:CCAGCTCCATGTGGTTATCCAT	111
GSTO1	Glutathione S-transferase omega 1	Fw:GAACGGCTGGAAGCAATGAAG Rev:TGCCATCCACAGTTTCAGTTT	69
GSTO2	Glutathione S-transferase omega 2	Fw:TGCCCCTATTCTCACAGGACC Rev:TCCAGGTACTCACAAGCAATAAC	188
GSTP1	Glutathione S-transferase pi 1	Fw:CCCTACACCGTGGTCTATTTCC Rev:CAGGAGGCTTTGAGTGAGC	137
GSTT1	Glutathione S-transferase theta 1	Fw:TCTACCTGACGCGCAAATATAAG Rev:CTTCTCCGCAGAGTCGTGT	112
GSTT2	Glutathione S-transferase theta 2	Fw:GACGCTCAAGGATGGTGATTT Rev:GCAGGTCAGATGGATACCAGT	104
HNMT	Histamine N-methyltransferase	Fw:GTGGAAAAAGTACGGATCACGC Rev:GTGGAAAAAGTACGGATCACGC	211
INMT	Indolethylamine N-methyltransferase	Fw:AAGGGGACACGCTGATTGAC Rev:AGTCGGAGAGAGTGATGTCTTG	93
MGST1	Microsomal glutathione S-transferase 1	Fw:ATGACAGAGTAGAACGTGTACGC Rev:TACAGGAGGCCAATTCCAAGA	82
MGST2	Microsomal glutathione S-transferase 2	Fw:TCGGCCTGTCAGCAAAGTTAT Rev:TGTTGTGCCCGAAATACTCTCT	119
MGST3	Microsomal glutathione S-transferase 3	Fw:GGCCCACCTAGCCATCAATG Rev:CGCTGAATGCAGTTGAAGATGT	111
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Fw:AGCCACATCGCTCAGACA Rev:GCCCAATACGACCAAATCC	69
RPLPO	Ribosomal protein, large, P0	Fw:CCTCATATCCGGGGGAATGTG Rev:GCAGCAGCTGGCACCTTATTG	95
ACTB	Actin, beta	Fw:CCAACCGCGAGAAGATGA Rev:CCAGAGGCGTACAGGGATAG	97

The complete sequence description of the target gene was obtained from the nucleotide sequence database (Primerbank)

Publication nº 3

Human hydroxytyrosol's absorption and excretion from a nutraceutical.

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Human hydroxytyrosol's absorption and excretion from a nutraceutical

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ABSTRACT

Among the various (poly)phenols that are being sold as such or as part of a more complex mixture, hydroxytyrosol (HT) is the only one that bears a European Food Safety Authority health claim. Therefore, several HT-based products are being developed and sold and it becomes necessary to evaluate its accessibility following ingestion. Twenty-one volunteers were recruited for a randomized, crossover, placebo-controlled, and double-blind intervention study. We performed a Latin square design: after one-week washout, i.e. olive-free diet, subjects were randomly assigned to the placebo (maltodextrin), 5, or 25 mg/day HT group. Twenty-four hour urine samples were collected after the intervention week, and baseline urines were collected the week before the study and during periods of washout. The results show that HT given as the foremost component of a nutraceutical preparation is bioavailable and is recovered in the urine chiefly as sulphate-3′.

1. Introduction

The nutraceutical and functional food market is rapidly expanding and several new products enter the market on a daily basis (Mahabir, 2014; Tome-Carneiro & Visioli, 2015). Of note, such products are rarely tested in controlled human trial settings and the efficacy of individual molecules or raw extracts is often questionable. In addition, the bioavailability of individual molecules or active principle(s) is seldom assessed, in part because of technical limitations and lack of proper equipment.

Among the various (poly)phenols that are being sold as such or as part of a more complex mixture, hydroxytyrosol (HT) is the only one that bears a European Food Safety Authority health claim (EFSA Panel on Dietetic Products, 2011). Therefore, several HT-based products are being developed and sold (Visioli & Bernardini, 2011) and it becomes necessary to evaluate accessibility of HT following ingestion. It is noteworthy that HT bioavailability has been reported after extra virgin olive oil

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administration (Caruso, Visioli, Patelli, Galli, & Galli, 2001; Miro-Casas et al., 2003), yet never after the intake of HT-containing supplements, with the exception of one study with pure HT (Gonzalez-Santiago, Fonolla, & Lopez-Huertas, 2010).

In this study, we report the urinary excretion of HT (as such and as its metabolites) after its administration to healthy volunteers.

2. Materials and methods

2.1. Standards and chemicals

Hydroxytyrosol (HT, 98% purity) standard was purchased from Extrasynthese (France). HT 3′-O- and 4′-O- glucuronides (HT-G-3′ and HT-G-4′, 86% and 97% purity, respectively) were synthesized as previously described (Giordano, Dangles, Rakotomanomana, Baracchini, & Visioli, 2015). HT 3′-O-sulphate (HT-S-3′, 98% purity) standard was bought from Toronto Research Chemicals Inc. (Toronto, ON, Canada). Hydroxyphenylpropanol (HOPhPr, 99% purity), used as the internal standard (ISTD), was purchased from Sigma-Aldrich (St. Louis, MO, USA).

LC-grade solvents methanol and ACN were purchased from Scharlau Chemie, S.A. (Sentmenat, Spain). Ammonium acetate and glacial acetic acid were purchased from Panreac Química, S.A.U. (Castellar del Vallés, Spain). Ultrapure water (Milli-Q) was obtained from Millipore (Bedford, MA, USA).

The capsules that we administered were elaborated from an olive mill waste water extract preparation called Hytolive®, supplied by the company Genosa ID, S.L. (Madrid, Spain).

2.2. Subjects and study design

The study protocol was approved by the local ethics committee and written informed consent was obtained from all subjects prior to starting the trial. This work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and is registered at ClinicalTrials.gov (identifier: NCT02273622).

Samples of this research were obtained from a previous intervention study, whose objective was to evaluate the effect of HT on the gene expression of Phase II enzymes (Crespo et al., 2015). Briefly, twenty-one volunteers were recruited for a randomized, crossover, placebo-controlled, and double-blind intervention study. The design of this study is shown in Fig. 1. We performed a Latin square design: after one-week washout, i.e. olive-free diet, subjects were randomly assigned to the placebo (maltodextrin) group, 5 mg/day HT group, or 25 mg/day HT (Hytolive®) group. Baseline characteristics of participants and inclusion and exclusion criteria are given in detail in Supplementary Information 1 (S.I.1 in Appendix S1). Volunteers were given dietary guidelines (Supplementary Information 2, S.I.2 in Appendix S1) that included abstention from olive products and limitation of high-polyphenol foods and alcohol (Crespo et al., 2015). Twenty-four hour urine samples were collected after the intervention week, and baseline urines were collected the week before the study and during periods of washout, and immediately stored at –80 °C.

2.3. Pretreatment and processing of the urine samples

A total of 63 24-hour (from 21 volunteers, collected in the three experimental phases, after administration of the supplement) and 42 basal urine samples (collected during the final days of the second and third washout periods) were analysed.

All urine samples were thawed, vortexed, and centrifuged at 9000 × *g* for 5 min at 4 °C. The supernatant (20 µL) from each urine sample was diluted with 0.1% acetic acid by a factor of 10 (1:10 vol:vol) for detection of HT and its glucuronidate metabolites and by a factor of 50 (1:50 vol:vol) for its sulphates (HT-S-3′and HT-S-4′). Calibration standards of 5-10-25-50-100-250-500-1000 ng/mL for HT and 20-40-100-200-400-1000-2000-4000 ng/mL for HT-G-3′, HT-G-4′ and HT-S-3′ in blank human urine were processed like the 10-fold diluted samples. An internal standard (HOPhPr) was used at the final concentration of 500 ng/mL in all cases. Samples and calibration curves were distributed in 96-well plates and 2 µL of each were injected in randomized order.

2.4. Sample analysis

LC–MS/MS analysis of diluted samples was performed on the Agilent (Santa Clara, CA, USA) 1290 Infinity Binary LC system coupled to an AB SCIEX QTRAP® 6500 spectrophotometer. Acquity UPLC BEH C18 1.7 µm, 2.1 × 5 mm analytical column (Waters) at 40 °C and 1 mM ammonium acetate at pH 5.0 and 100% ACN as aqueous (A) and organic (B) mobile phases, respectively, were used for separation (Khymenets et al., 2011; Kotronoulas et al., 2013). Next, gradient elution (B% (v/v), t (min)) at flow of 0.4 mL/min was applied: (1%, 0–3); (1–20%, 3–3.2); (20%, 3.2–4.5); (20–95%, 4.5–4.8); (95%, 4.8–5.3); (95–1%, 5.3–5.5); (1%, 5.5–6.5). Common MS parameters were as follows: ion spray voltage (IS) –4500.00, source temperature (TEM) 600 °C, curtain

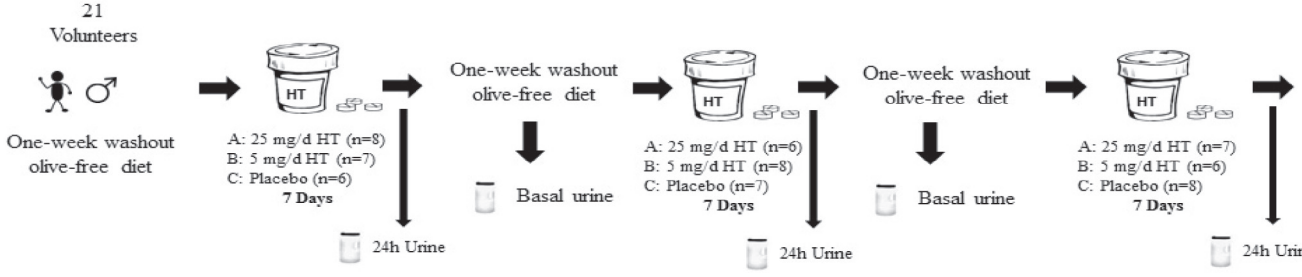

Fig. 1 – Study design.

gas (CUR) 20.00 psi, ion source gas 1 (GS1) and gas 2 (GS2) 50.00 psi each, collision-activated dissociation (CAD) 3.00 psi, entrance potential (EP) –10.00 and cell exit potential (CXP) 13.00. The data were collected under negative ionization in multiple reaction monitoring mode (MRM) with following settings for compound fragmentations (declustering potential, DP: V; collision energy, CE: eV): HT $153^- \rightarrow 123^-$ (DP: –55; CE: –20); HT-G-3′ and HT-G-4′ $329^- \rightarrow 153^-$ (DP: –60; CE: –30); HT-S-3′ and HT-S-4′ $233^- \rightarrow 153^-$ (DP: –60; CE: –25) and HOPhPr $151^- \rightarrow 121^-$ (DP: –65; CE: –22). HT, HT-G-3′, HT-G-4′ and HT-S-3′ were quantified using calibration curves constructed with corresponding standards. HT-S-4′ has been identified only in samples with high concentration of HT-S-3′; its concentration was estimated using slope of HT-S-3′ calibration curve. The method based on LC–MS/MS analysis for HT and its glucuronidated and sulphated metabolites in diluted urine samples was successfully validated, showing good linearity ($r^2 \geq 0.99$ in all cases) and following sensitivity (LOQs): 5 and 20 ng/mL urine for HT and its metabolites (glucuronides and sulphate), respectively. Intra- and inter-day precision and accuracy results were according to the standard requirements (U.S. Department of Health and Human Services, 2001) for method validation criteria: RSD% and ERR% were <20% (except HT-S-3′, where they were ≤28%, due to the impact of ever existing endogenous metabolite) for low and <15% (all compounds) for medium and high concentrations of tested standards.

The results were processed using Analyst 1.6.2 Software (AB SCIEX) and then statistically analysed. The final results, expressed as concentrations (ng/mL urine) of HT, HT-G-3′, HT-G-4′, HT-S-3′ and HT-S-4′, are shown in Supplementary Table 1 (S.T.1 in Appendix S1).

2.5. *Statistical analysis*

Data were analysed with R Statistical Software version 3.1.1. Continuous descriptive variables were expressed as means ± SEM. Two-way repeated measures ANOVA was used to evaluate the effects of time (basal and 24-hour urine), treatment (A, B, C) and the time × treatment interaction. A Bonferroni correction for multiple analyses was applied and models were adjusted for age and sequence (ABC/CAB/BCA) as covariates. All statistical analyses were considered as bilateral and significance was set at $p < 0.05$.

3. Results

The administration of a standardized, 10%-HT nutraceutical resulted in a dose-dependent urinary excretion of HT and its metabolites (Table 1). These changes were statistically significant and were more pronounced for HT-S-3′. Of note, this molecule was also detected in urines from placebo-treated subjects, possibly as a consequence of endogenous HT production and excretion (Perez-Mana et al., 2015a,b). Inter-individual variability varied, but was – on average – ~10%.

Quantitatively, the total amount of HT and its metabolites recovered in the urine accounted for 21% (for the 25 mg dose) to 28% (for the 5 mg dose) of the administered dose (Table 2).

Table 1 – Changes in urinary concentration of hydroxytyrosol and its main metabolites during the study.

	A			B			C			ANOVA*		
	n = 21			n = 21			n = 21					
	ng/mL[a]	b		ng/mL[a]	b		ng/mL[a]	b		a	b	c
		Initial	Final		Initial	Final		Initial	Final			
HT	3.65 (0.85)	0 (0)	2e-06 (6.9e-07)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.0048*	0.0005*	0.0005*
HT-G-4	375.5 (40.0)	8.5e-06 (2.7e-06)	2e-04 (2.3e-05)	82.95 (10.84)	8.7e-06 (1.6e-06)	4.5e-05 (5.4e-06)	18.5 (5.94)	6.3e-06 (1.2e-06)	1.6e-05 (8.5e-06)	<0.0001*	<0.0001*	<0.0001*
HT-G-3	588.1 (57.4)	1.5e-05 (2.7e-06)	0.00031 (3.3e-05)	103.4 (11.15)	1.3e-05 (1.6e-06)	5.6e-05 (6.3e-06)	12.9 (2.03)	1.2e-05 (1.7e-06)	9.2e-06 (2.8e-06)	<0.0001*	<0.0001*	<0.0001*
HT-S-3	3279 (311.3)	9.5e-05 (1.7e-05)	0.0017 (0.00016)	932.3 (122.2)	9.3e-05 (1.7e-05)	0.00051 (7e-05)	117.9 (36.4)	0.00011 (2.3e-05)	8.7e-05 (3.3e-05)	<0.0001*	<0.0001*	<0.0001*
HT-S-4	52.29 (4.59)	0 (0)	2.8e-05 (2.7e-06)	10.71 (1.98)	0 (0)	5.6e-06 (1e-06)	0.67 (0.67)	0 (0)	4.3e-07 (4.3e-07)	<0.0001*	<0.0001*	<0.0001*

A: 250 mg Hytolive (25 mg hydroxytyrosol); B: 50 mg Hytolive (5 mg hydroxytyrosol); C: placebo.
n: number of volunteers studied by treatment group.
HT (free hydroxytyrosol); HT-G-4 (hydroxytyrosol-O-glucuronide 4); HT-G-3 (hydroxytyrosol-O-glucuronide 3); HT-S-3 (hydroxytyrosol-sulphate-3); HT-S-4 (Hydroxytyrosol-sulphate-4).
a: Time effect, the evolution in each group from beginning to end of the intervention; b: Differences between treatments (A, B, C) independent of time; c: Differences in evolution between groups as a result of treatment (A, B, C).
[a]Concentration of compound in ng/mL after corresponding treatment; [b]Normalized by creatinine concentrations for each individual group before and after treatment. Data are means (SEM).
* $P < 0.05$.

Table 2 – Twenty-four hour urine excretion of hydroxytyrosol and its main metabolites (as milligrams).

Dose (mg)	HT (mg)	HT (%)	HT-G-4 (mg)	HT-G-4 (%)	HT-G-3- (mg)	HT-G-3 (%)	HT-S-3 (mg)	HT-S-3 (%)	HT-S-4 (mg)	HT-S-4 (%)
0	0.00	0.00	0.03	0.00	0.02	0.00	0.14	0.00	0.00	0.00
5	0.00	0.00	0.11	2.23	0.14	2.78	1.18	23.1	0.01	0.26
25	0.00	0.02	0.46	1.83	0.72	2.87	4.15	16.6	0.07	0.28

0: Placebo; 5: 5 mg hydroxytyrosol; 25: 25 mg hydroxytyrosol.
HT: free hydroxytyrosol; HT-G-4: hydroxytyrosol-O-glucuronide 4; HT-G-3: hydroxytyrosol-O-glucuronide 3; HT-S-3: hydroxytyrosol-sulphate-3; HT-S-4: hydroxytyrosol-sulphate-4.

Table 3 – Twenty-four hour urine excretion of hydroxytyrosol and its main metabolites (as micromoles).

Dose (μM)	HT (μM)	HT (%)	HT-G-4 (μM)	HT-G-4 (%)	HT-G-3- (μM)	HT-G-3 (%)	HT-S-3 (μM)	HT-S-3 (%)	HT-S-4 (μM)	HT-S-4 (%)	Total (μM)	Total excreted%
0	0.00	0.00	0.02	0.00	–0.01	0.00	–0.26	0.00	0.00	0.00	–0.05	0.00
32.4	0.00	0.00	0.22	0.67	0.27	0.84	3.47	10.7	0.04	0.14	0.80	12.4
162	0.02	0.01	1.10	0.68	1.72	1.06	13.5	8.33	0.22	0.13	3.32	10.2

0: Placebo; 3.2 E-5 μM: 5 mg hydroxytyrosol; 16 E-5 μM: 25 mg hydroxytyrosol.
HT: free hydroxytyrosol; HT-G-4: hydroxytyrosol-O-glucuronide 4; HT-G-3: hydroxytyrosol-O-glucuronide 3; HT-S-3: Hydroxytyrosol-sulphate-3; HT-S-4: Hydroxytyrosol-sulphate-4.
μM: micromole; Total
(μM): Total micromole as the sum of compounds found in urine samples; Total excreted
(μM): Total percentage as the sum of compounds recovered in urine.

Again, the major metabolite we detected was HT-S-3′, which accounted for 23.6% (for the 5 mg dose) to 16.6% (for the 25 mg dose) of the administered HT.

Quantitatively, as we represent in Table 2, the total amount of HT recovered in the urine was minimal and accounted for 0.02% (only for the 25 mg dose). For others metabolites, we observed a dose-dependent increase in their excretion. Again, the major metabolite we detected was HT-S-3′, which accounted for 23.1% (for the 5 mg dose) and 16.6% (for the 25 mg dose) of the administered HT, followed by HT-G-3′ with 2.78% (for the 5 mg dose) and 2.87% (for the 25 mg dose).

When results were expressed as micromole% (in order to compare the different excreted compounds; Table 3), the total per cent excretion of all components dropped to 12.4% (for the 5 mg dose) and 10.2% (for the 25 mg dose). The per cent excretion of HT-S-3′ dropped to 10.7% (for the 5 mg dose) and 8.33% (for the 25 mg dose) of the initial dose, but this metabolite remained the most abundant one we recovered.

4. Discussion

One important – yet often overlooked issue – in the nutraceutical field is that of absorption and/or bioavailability of the active principle(s). This applies to omega 3 fatty acids, vitamins, and (poly)phenols. We here report that HT (one of the most popular and biologically active phenol) is absorbed and excreted when given as an olive mill waste water extract preparation. In particular, we recovered ~8 to 10% (as mole%) of the administered HT in the urine and confirmed that most of it undergoes sulphation at the 3′ position. To date, only one study has been published with pure HT (Gonzalez-Santiago et al., 2010), whereas many other ones report excretion of this phenol when given as component of extra virgin olive oil to rats or humans. Indeed, there is ample evidence of the absorption and excretion of HT *via* extra virgin olive oil use, even though a comprehensive profile of its metabolites is being slowly developed. In the first report, Visioli et al. (2000) described how 30–60% of the administered HT was recovered in the urine, mostly as glucuronide conjugate. These data were subsequently confirmed by Vissers, Zock, Roodenburg, Leenen, and Katan (2002). Afterwards, more complete investigations (Miro-Casas et al., 2003) contributed to the near-complete elucidation of HT's metabolism in humans. More recently, HT sulphate has been proposed as a suitable biomarker for monitoring compliance with olive oil intake as its values in plasma or/and 24-h urine were significantly higher after extra virgin olive oil administration compared to baseline pre-intervention concentrations (Rubió et al., 2014). The data we present here reinforce this notion: HT-S-3′ should be quantified in studies of HT as nutraceutical, to monitor compliance.

One unresolved issue is whether the extensive first-pass metabolism affects the manifold *in vitro* activities reported for HT and (poly)phenols in general. Indeed, this is an often overlooked aspect of (poly)phenol research and calls for more metabolite-based biochemical and molecular studies (Giordano et al., 2015), even though organ-specific deconjugation might, theoretically, yield pure HT and contribute to its biological activities (Giordano et al., 2015).

In conclusion, we prove that HT given as the foremost component of a nutraceutical preparation is bioavailable and is recovered in the urine chiefly as sulphate-3′, which can be adopted as biomarker of extra virgin olive oil consumption. This is important in light of future HT-based nutraceutical formulations and epidemiological studies.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2016.02.046.

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SUPPLEMENTARY INFORMATION
Supplementary materials and methods

S.I.1 Inclusion and exclusion criteria

Volunteers included in this trial were age between 20 and 40 years; adequate understanding of the study; willingness to complete the entire treatment. Exclusion criteria included body mass index <19 or >26; diagnosis of diabetes mellitus, hypertension, dyslipidemia or other cardiometabolic disorders; impaired cognitive function; diagnosed hepatic, renal, or cardiovascular disease; allergy to olives and their derivatives; pharmacological therapies; and habitual smoking.

S.I.2. Dietary guidelines

Food	Variety	Guideline
Olives	Green or black	They should **not be consumed** along the whole intervention
products made from or with olives	Derived from olives, pates, jams vinaigrettes Pizza with olives Olive bread	
Olive oil		
products made from or with olive oil	Mayonnaise with olive oil, biscuits, fried	
Cod-liver oil		
Hydroxytyrosol supplements		
Supplements of vitamins, minerals and antioxidants		
High content in polyphenols and antioxidants	Red wine, wine, beer, coffee, tea, fruit, chocolate, fruits, nuts.	They may only be consumed in **moderate amounts**

S.T.1. Quantitative results of the samples studied. Concentrations of free HT and its principal metabolites.

Voluntary ID	Group	Initial HT ng/mL	Final HT ng/mL	Initial HT´-G-4´ ng/mL	Final HT-G-4´ ng/mL	Initial HT-G-3´ ng/mL	Final HT-G-3´ ng/mL	Initial HT-S-3´ ng/mL	Final HT-S-3´ ng/mL	Initial HT-S-4´ ng/mL*	Final HT-S-4´ ng/mL*	24 h Urine mL	Basal Urine mL
856	A	0.0	12.0	0.0	482.5	25.3	712.0	28.6	1811.2	0.0	17.1	1250.0	42.5
857	A	NA	0.0	NA	124.3	NA	259.3	NA	2466.9	NA	40.4	1600	NA
858	A	0.0	5.2	*12.9*	140.4	20.9	283.8	98.6	4385.9	0.0	94.5	1820.0	42.5
859	A	NA	6.8	NA	840.0	NA	764.6	NA	1670.2	NA	22.6	1100	NA
860	A	0.0	0.0	0.0	394.9	*14.7*	557.9	0.0	2830.5	0.0	51.3	1450.0	47.5
861	A	0.0	5.2	*15.8*	554.0	36.4	1113.8	42.7	3091.9	0.0	53.4	825.0	50.0
862	A	NA	10.0	NA	552.9	NA	901.8	NA	5449.9	NA	80.5	800	NA
864	A	NA	0.0	NA	218.6	NA	251.8	NA	2960.7	NA	54.3	1920	NA
865	A	0.0	0.0	0.0	228.2	33.0	377.9	117.4	2185.5	0.0	36.1	1950	51.0
866	A	0.0	0.0	0.0	320.0	*17.8*	506.7	431.9	4813.1	0.0	73.1	1200	42.5
867	A	0.0	5.2	36.7	418.0	101.5	752.7	282.9	4827.7	0.0	36.8	800	45.0
868	A	NA	0.0	NA	251.0	NA	365.0	NA	1650.5	NA	28.9	1850	NA
869	A	NA	0.0	NA	182.4	NA	286.9	NA	2479.0	NA	48.7	1300	NA
870	A	0.0	6.7	55.3	639.0	70.9	1049.9	695.2	3849.4	0.0	62.1	800	42.5
871	A	NA	0.0	NA	185.2	NA	302.9	NA	1497.8	NA	28.8	3000	NA
872	A	NA	5.9	NA	289.0	NA	483.4	NA	2320.5	NA	53.1	1600	NA

873	A	0.0	7.0	0.0	478.5	*16.7*	717.0	46.3	5214.9	0.0	75.4	750	42.5
874	A	0.0	0.0	*12.3*	247.8	*15.5*	456.5	83.0	1878.0	0.0	54.6	1850	40.0
875	A	0.0	6.9	0.0	475.6	*10.7*	689.8	72.4	5693.3	0.0	88.0	1000	55.0
876	A	0.0	0.0	109.1	384.0	37.9	647.1	492.3	5133.6	0.0	45.1	1350	45.0
877	A	0.0	5.8	0.0	480.1	0.0	871.0	67.4	2653.4	0.0	53.2	1150.0	47.5
856	B	0.0	0.0	0.0	103.6	*15.8*	140.2	0.0	792.9	0.0	10.3	1150.0	52.5
857	B	0.0	0.0	24.4	43.1	*17.1*	99.9	22.8	963.1	0.0	16.7	1600.0	45.0
858	B	NA	0.0	NA	82.3	NA	148.0	NA	482.9	NA	12.0	2000	NA
859	B	0.0	0.0	23.9	208.4	*18.5*	123.8	150.4	730.4	0.0	10.2	1430.0	50.0
860	B	0.0	0.0	30.1	185.5	21.8	209.3	226.6	1255.2	0.0	16.9	1100.0	45.0
861	B	NA	0.0	NA	90.3	NA	127.9	NA	1433.9	NA	25.4	950	NA
862	B	0.0	0.0	0.0	97.2	*14.9*	211.4	75.2	1178.3	0.0	14.7	600.0	42.5
864	B	0.0	0.0	*10.7*	59.0	30.9	54.7	213.0	882.0	0.0	11.5	1900.0	47.0
865	B	0.0	0.0	35.4	100.3	29.6	102.2	544.2	953.4	0.0	13.7	1350.0	51.0
866	B	0.0	0.0	*15.0*	30.1	28.2	49.9	175.4	530.8	0.0	0.0	1750.0	40.0
867	B	NA	0.0	NA	65.5	NA	74.3	NA	1781.0	NA	22.6	1250	NA
868	B	0.0	0.0	29.5	62.0	31.4	38.1	461.5	451.3	0.0	0.0	2150.0	45
869	B	0.0	0.0	*18.6*	20.8	*13.8*	27.1	179.0	259.7	0.0	0.0	2550.0	48
870	B	0.0	0.0	*19.0*	133.1	32.5	155.9	222.5	522.0	0.0	0.0	1100.0	45.0
871	B	0.0	0.0	0.0	45.3	*13.6*	62.2	25.3	711.7	0.0	13.2	2100.0	46.0
872	B	0.0	0.0	*11.4*	29.9	0.0	44.7	79.4	283.8	0.0	0.0	2430.0	51
873	B	NA	0.0	NA	134.7	NA	119.0	NA	2325.4	NA	22.5	650	NA
874	B	NA	0.0	NA	62.3	NA	100.5	NA	191.8	NA	0.0	2600	NA

875	B	NA	0.0	NA	72.7	NA	100.9	NA	1472.9	NA	26.0	1000	NA
876	B	NA	0.0	NA	84.1	NA	103.2	NA	1678.0	NA	9.3	1250	NA
877	B	0.0	0.0	0.0	31.8	*14.6*	78.1	142.3	699.1	0.0	0.0	1300	42.5
856	C	NA	0.0	NA	98.2	NA	33.5	NA	320.8	NA	0.0	1250	NA
857	C	0.0	0.0	35.6	0.0	102.4	*18.6*	1258.0	133.6	0.0	0.0	1450	42.5
858	C	0.0	0.0	25.6	0.0	26.5	*19.5*	326.4	734.6	0.0	14.0	1330	47.5
859	C	0.0	0.0	24.8	44.1	*17.6*	*15.9*	202.6	33.4	0.0	0.0	1300	50
860	C	NA	0.0	NA	52.8	NA	*17.3*	NA	25.6	NA	0.0	1850	NA
861	C	0.0	0.0	0.0	0.0	20.0	*12.5*	46.3	0.0	0.0	0.0	1500.0	51.0
862	C	0.0	0.0	27.9	0.0	28.8	*19.8*	109.1	170.6	0.0	0.0	850	45
864	C	0.0	0.0	23.3	45.9	20.5	*16.2*	106.4	32.8	0.0	0.0	1600	47.5
865	C	NA	0.0	NA	*17.3*	NA	*18.9*	NA	49.7	NA	0.0	2150	NA
866	C	NA	0.0	NA	0.0	NA	*14.9*	NA	49.3	NA	0.0	1650	NA
867	C	0.0	0.0	*12.8*	0.0	32.3	*14.6*	407.8	234.5	0.0	0.0	1100	48
868	C	0.0	0.0	*18.6*	0.0	*13.6*	0.0	26.8	0.0	0.0	0.0	1600.0	47.5
869	C	0.0	0.0	0.0	0.0	*16.4*	0.0	160.8	0.0	0.0	0.0	2350.0	52.5
870	C	NA	0.0	NA	*14.9*	NA	*19.9*	NA	202.6	NA	0.0	1050	NA
871	C	0.0	0.0	0.0	0.0	*17.7*	*15.1*	275.7	91.2	0.0	0.0	2250	45
872	C	0.0	0.0	*11.8*	31.1	*11.1*	0.0	113.0	*14.1*	0.0	0.0	1750.0	47.0
873	C	0.0	0.0	29.2	0.0	20.2	0.0	195.3	0.0	0.0	0.0	1450.0	47.5
874	C	0.0	0.0	0.0	0.0	*18.0*	0.0	112.4	63.0	0.0	0.0	1700.0	45
875	C	0.0	0.0	34.4	0.0	*14.1*	0.0	246.4	33.5	0.0	0.0	1430.0	40
876	C	0.0	0.0	0.0	57.9	*12.9*	*19.5*	112.3	171.1	0.0	0.0	1365.0	40

877	C	NA	0.0	NA	26.4	NA	*15.4*	116.5	116.5	NA	0.0	440	NA

A: 250 mg Hytolive (25 mg hydroxytyrosol); B: 50 mg Hytolive (5 mg hydroxytyrosol); C: Placebo. HT (free Hydroxytyrosol); HT-G-4´ (Hydroxytyrosol 4´-*O*-glucurodine); H-G-3´ (Hydroxytyrosol 3´-O-glucurodine); HT-S-3 (Hydroxytyrosol 3´-*O*-sulphate); HT-S-4´ (Hydroxytyrosol 4´-*O*-sulphate). Initial: result obtained in basal urine samples; Final: Result obtained in 24 h urine samples. ng/mL: nanogram/millilite

Publication nº 4

Hydroxytyrosol supplementation modulates the expression of miRNAs in rodents and in humans.

Tomé-Carneiro J*, **Crespo MC***, Iglesias-Gutierrez E, Martín R, Gil-Zamorano J, Tomas-Zapico C, Burgos-Ramos E, Correa C, Gómez-Coronado D, Lasunción MA, Herrera E, Visioli F, Dávalos A.

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Hydroxytyrosol supplementation modulates the expression of miRNAs in rodents and in humans

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Abstract

Dietary microRNAs (miRNAs) modulation could be important for health and wellbeing. Part of the healthful activities of polyphenols might be due to a modulation of miRNAs' expression. Among the most biologically active polyphenols, hydroxytyrosol (HT) has never been studied for its actions on miRNAs. We investigated whether HT could modulate the expression of miRNAs *in vivo*. We performed an unbiased intestinal miRNA screening in mice supplemented (for 8 weeks) with nutritionally relevant amounts of HT. HT modulated the expression of several miRNAs. Analysis of other tissues revealed consistent HT-induced modulation of only few miRNAs. Also, HT administration increased triglycerides levels. Acute treatment with HT and *in vitro* experiments provided mechanistic insights. The HT-induced expression of one miRNA was confirmed in healthy volunteers supplemented with HT in a randomized, double-blind and placebo-controlled trial. HT consumption affects specific miRNAs' expression in rodents and humans. Our findings suggest that the modulation of miRNAs' action through HT consumption might partially explain its healthful activities and might be pharmanutritionally exploited in current therapies targeting endogenous miRNAs. However, the effects of HT on triglycerides warrant further investigations.

Keywords: Hydroxytyrosol; Intestine; miRNAs; Dietary Supplementation; Lipids

1. Introduction

MicroRNAs (miRNAs) exert important regulatory actions on gene expression not only under physiological circumstances but also in disease [1]. Indeed, miRNAs often target multiple functionally related genes and extensively interfere with biological processes, rendering them good candidates for therapeutic and/or nutraceutical interventions [2]. Two divergent approaches are currently being studied: (1) miRNAs inhibition *via* pharmaceutical formulations and (2) the use of miRNAs mimics in therapy.

To therapeutically constraint miRNAs, small-molecule inhibitors or activators aimed at miRNAs' expression regulation are being actively studied. Within this context, the potential role of plant-derived phenolic molecules (*e.g.* polyphenols) in miRNAs modulation is being very actively investigated [3–6]. As of now, however, there is no clear published evidence of a definite direct effect of a polyphenol on a specific miRNA. Therefore, phenolic modulation of miRNAs becomes an attractive strategy to target several biological processes and, in turn, ameliorate prognosis [7].

Olive phenolics exhibit cardioprotective activities such as their ability to protect low-density lipoproteins from oxidation (European Food Safety Authority scientific opinion). In particular, hydroxytyrosol (HT), the foremost phenolic component of extra virgin olive oil, has been hypothesized to possess antiinflammatory, antiaggregant and anticancer properties, among others [8]. Nevertheless, the exact molecular mechanisms underlying many of these actions are yet to be fully clarified [9]. Regarding miRNAs, previous *in vitro* studies suggest that *Olea europaea* leaf extracts alter some cancer-related miRNAs in glioblastoma cells [10].

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In addition, the Mediterranean diet (of which extra virgin olive oil is an important component) has been reported to modify inflammation-related miRNAs [11].

In this study, we aimed to explore the effects of nutritionally relevant amounts of HT on miRNAs' modulation *in vivo*.

2. Materials and methods

2.1. Materials

HT was kindly donated by Seprox Biotech (Madrid, Spain); miScript II RT for RT-qPCR and Qiazol were purchased from Qiagen (Izasa, Barcelona, Spain). The primers used in the validation of our experiments were purchased from ISOGEN (Life Science, Belgium).

2.2. Animals and diets

This investigation conforms to the Guide for the Care and Use of Laboratory Animals, published by the US National Research Council (Eight Edition, 2010) and was approved by the Animal Experimentation Committee of the Universidad Complutense de Madrid (CEA-UCM 93/2012), Universidad de Oviedo and Hospital Universitario Ramón y Cajal (CEBA-HRC 23/2011). For the long-term feeding studies, young C57BL/6 mice from two different cohorts (2 months old, $n=14$: first cohort and $n=20$: second cohort) were acclimatized and kept on a 12:12 light/dark cycle, with the period of darkness between 7:00 p.m. and 7:00 a.m., for at least 1 week before the beginning of the experimentation. During this period, mice were fed a standard chow diet; food and water were given *ad libitum*. Thereafter, mice were maintained for 8 weeks under two different diet regimens (Research Diets, Inc., New Brunswick, NJ): (1) purified control diet ($n=17$) or (2) purified control diet added with 0.03 g% HT ($n=17$). This dose closely approximates human intake [8] and is a very low one once body surface area is taken into account [12]. Each diet provided 24.0%, 15.0% and 61.0% kcal from protein, fat and carbohydrates, respectively [13]. To reduce diurnal variations, animals were sacrificed between 10:00 and 11:00 a.m. Mice were anesthetized with ketamine (0.4 mg/ml) and dissected through a midline incision in the abdomen. Blood samples were collected through cardiac puncture. Phosphate-buffered saline (PBS; pH 7.4) was perfused through the portal vein for 1 min to remove blood. All tissues were extracted quickly, washed in PBS, frozen with liquid nitrogen and stored at −80°C.

For the acute ingestion studies, we used 36 young male C57BL/6 mice of 10 weeks of age. Animals were maintained under the same conditions of acclimatization described above and with a standard diet until the time of the experiment. Animals were separated into four study groups ($n=9$ per group). The day of experiment, after 6 h fasting, mice were provided, by gavage, with 15 mg of HT dissolved in water and then sacrificed at different time points: 0 h (control), 1 h, 2 h and 4 h, using the same methodology as for long-term feeding. Blood samples were collected through cardiac puncture, centrifuged at 1500*g* for 15 min for plasma collection, snap-frozen and stored at −80°C. In addition, after perfusion, liver and intestines samples were extracted, washed with PBS, snap-frozen and kept at −80°C.

2.3. Lipid analysis

Total plasma cholesterol, triglyceride and phospholipid concentrations were analyzed with a microtiter assay using commercially available kits (Bradford Diagnostics, Kemia Científica S.A., Spain). For plasma lipoprotein profiles, 200 μl of pooled plasmas from each dietary group was subjected to fast protein liquid chromatography (FPLC) gel filtration using a Superose 6 HR 10/30 column (Pharmacia). Samples were eluted with 150 mM NaCl, 10 mM Tris–HCl, 2 mM Na_2-EDTA and 0.02% NaN_3, pH 7.4, at a flow rate of 0.3 ml/min to collect 0.4-ml fractions. In individual fractions, cholesterol and triglyceride concentrations were determined using commercially available kits. For the analysis of lipids in tissues, the former were extracted with chloroform-methanol [14] and analyzed enzymatically using commercial kits from Spinreact (Sant Esteve de Bas, Spain) after being solubilized in water by the addition of Triton X-100 as described [15].

2.4. RNA extraction and microarray sample preparation

Total RNA from all tissues was isolated using Qiazol Lysis Reagent and miRNeasy Mini kit columns (Qiagen, Madrid, Spain). RNA was quantified using a NanoDrop-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Spain) and purity was assessed by measuring the 260/280 nm and 260/230 nm ratios. RNA integrity was assessed using Agilent's 2100 Bioanalyzer. Samples with RIN >8 were selected for analyses.

2.5. miRNA screening

For the unbiased whole genome miRNA screening, cDNA was synthesized from total RNA (40 ng) using Universal cDNA synthesis kit II (Exiqon). miRNA quantification was performed by real-time PCR (RT-qPCR) using the ExiLENT SYBR green master mix kit (Exiqon) on a 7900HT fast real-time PCR system (Applied Byosystems) using a 384-well plate format. Five samples per condition were analyzed using the mouse miRNome panels (752 mice mature miRNAs) Version 3 (Exiqon). miRNA relative expression analysis was performed using the GenEx software (MultiD Analyses AB, Sweden).

2.6. Microarray analysis

RNA amplification and labeling was carried out by using the Low RNA Input Linear Amplification Kit, PLUS, Two-Color (Agilent Technologies, Palo Alto, CA). Briefly, for each sample, 2 μg of total RNA input was amplified in two rounds of amplification by following manufacturer's instructions. The first strand cDNA syntheses and amplification reactions were carried out by using random and T7 primers, respectively. During the 2-h *in vitro* transcription, Cy3- or Cy5-labeled CTP was incorporated in each amplified RNA (cRNA) from reference pooled and tested samples, respectively. Reaction products were then purified using RNAeasy mini spin columns (Qiagen, Dusseldorf, Germany). Hybridization and slide and image processing procedures were all carried out according to the manufacturer's instructions (Two-Color Microarray-Based Gene Expression Analysis protocol). cRNA samples were fragmented at 60°C for 30 min and hybridized at 65°C for 17 h. Slides were scanned at a 10-μm resolution with Agilent G2565BA Microarray Scanner (Agilent Technologies, Palo Alto, CA). Signal quantification was carried out with Feature Extraction 9.1 software (Agilent Technologies, Palo Alto, CA) using default analysis parameters for Agilent's 44K whole genome mouse gene expression arrays (Feature Extraction protocol 44K). The reproducibility of the microarray data was assessed by performing a Pearson correlation coefficient analysis of the two biological replicates using the statistical computing program R (version 2.10.1; http://www.r-project.org/) between the absolute signal intensity of the background-corrected Cy5 channel (treated samples) and the absolute signal intensity of the background-corrected Cy3 channel (pool of control samples) for all the probes present on the microarray. The quality and homogeneity of the background-corrected and VSN-normalized microarray data for the Cy5 and Cy3 channels was assessed by the Bioconductor package arrayQualityMetrics version 2.4.3

Data were analyzed by using the GeneSpring Software (Agilent), following the manufacturer's instructions. Significantly modulated genes were defined as those with an absolute fold change higher than 1.5 in both directions and with an adjusted *P* value lower than .05. Differentially expressed genes were classified according to their functional role(s) in cellular or metabolic biological processes using the online GeneCodis analysis software for functional enrichment analysis [16].

Volcano plots represent the relationship between fold change and statistical significance of the differentially expressed genes, using an absolute fold change cutoff of 1.5. $\log_2$ fold change and $-\log_{10}$ adjusted *P* value are shown on the *x*-axis and *y*-axis, respectively. Each probe is represented by a dot. Colored dots represent statistically significant probes (adjusted *P* value <.05) with a $\log_2$ fold change either higher than 1 in both directions. A total of 342 probes were found to be significantly up-regulated, and 332 probes were found to be significantly down-regulated. These 674 differentially modulated genes were used for genetic interaction (GI) analysis (see below).

2.7. GI analysis

For GI analysis, a subset of miRNAs was selected from those that were significantly changed by HT supplementation. Only those miRNAs conserved within humans and showing a higher differentially expression pattern were included. A total of 21 miRNAs were selected from which three were down-regulated and 18 were up-regulated. For GI analysis, miRNA's targets were predicted using the miRanda algorithm [17]. When available, miRNAs' predicted targets were directly retrieved from the database (http://www.microrna.org/). For the selected miRNAs that were unavailable in the database (*e.g.* mmu-miR-1247-5p) target predictions were generated locally by running the miRanda algorithm code. Only targets showing a statistically significant differential

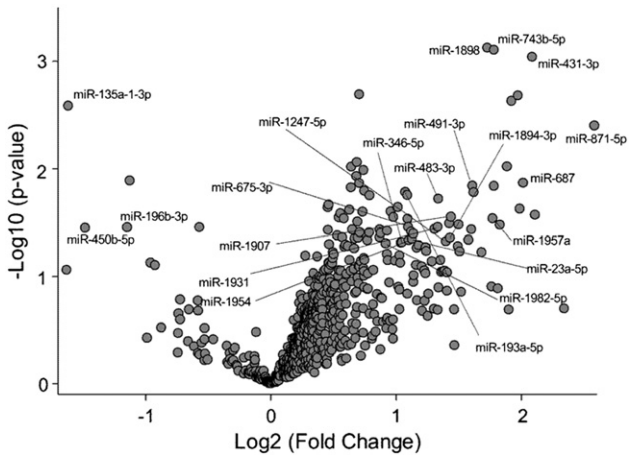


Fig. 1. HT supplementation modulates miRNAs expression. Volcano plot of hydroxytyrosol-modulated miRNAs in mouse small intestinal. miRNA expression was analyzed by RT-qPCR ($n=5$ mice per group). Labeled miRNAs are selected miRNAs for further studies.

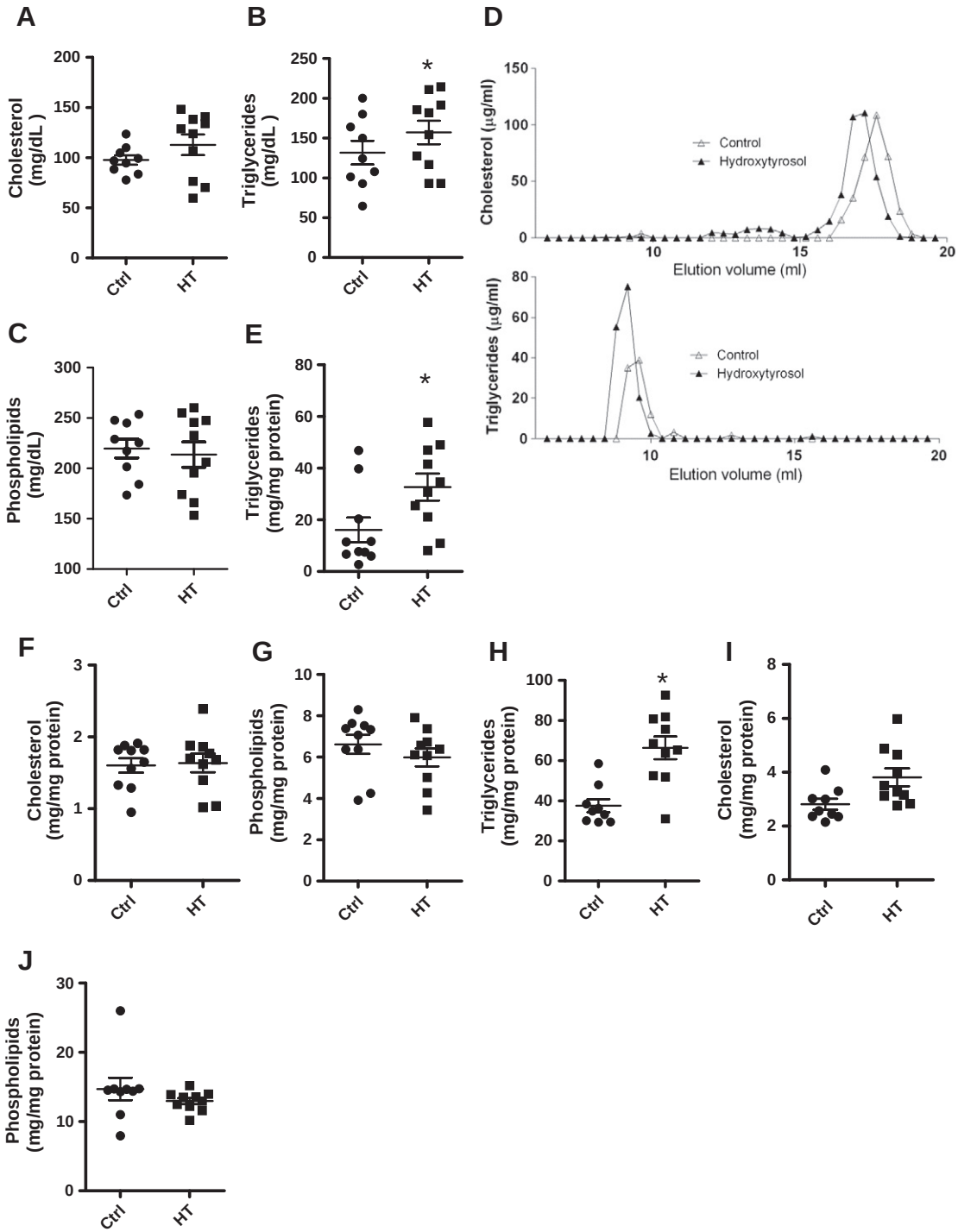
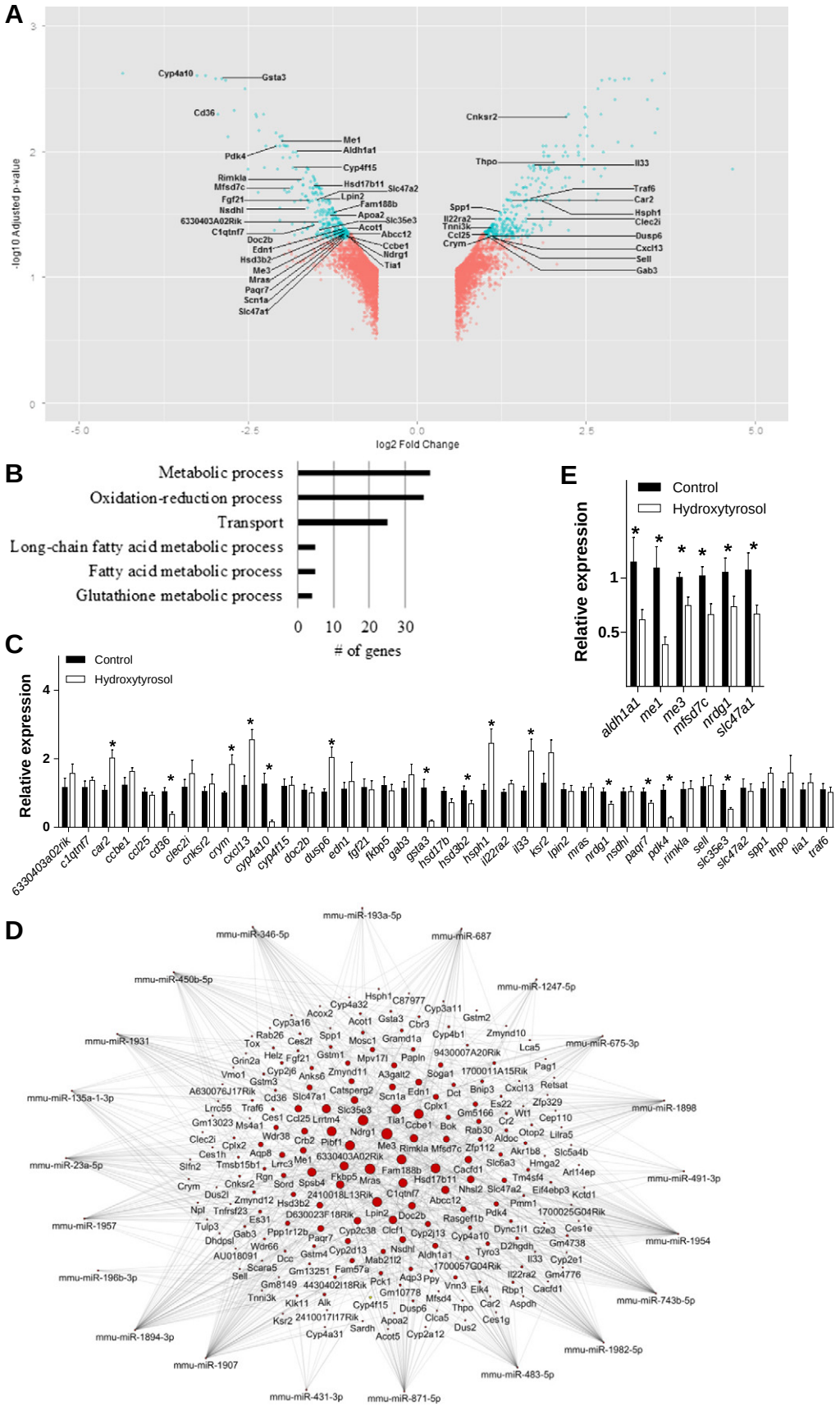


Fig. 2. Effects of HT supplementation on lipid metabolism. Plasma concentrations of cholesterol (A), triglycerides (B) and phospholipids (C) of mice receiving either chow diet (Ctrl) or HT supplementation during 8 weeks. (D) Plasma lipoprotein profile (FPLC) from mice following the different experimental conditions. Representative pooled plasma samples were fractionated and cholesterol and triglycerides were analyzed. Intestinal concentrations of triglycerides (E), cholesterol (F) and phospholipids (G). Liver concentrations of triglycerides (H), cholesterol (I) and phospholipids (J). $n=9$ in control and $n=10$ in HT-treated mice. *$P<.05$ *vs.* control without HT.

Fig. 3. miRNA–gene interactions contribute to intestinal HT-modulated genes. (A) Volcano plot of HT-modulated genes in mouse small intestine. Mice received either chow or HT-supplemented diet (8 weeks) and transcriptomic analysis evaluated. Significant modulated genes ($P<.05$) are depicted in green. (B) Functional enrichment analysis of biological processes. (C) Validation of selected modulated genes by RT-qPCR. *, significantly different from controls at $P<.05$. (D) GI analysis between modulated miRNAs and their predicted target genes from modulated genes. Target dot sizes are directly correlated with the number of interactions with the miRNA's set. (E) Validation of selected GI genes by RT-qPCR. *, significantly different from controls at $P<.05$.


A
-log10 Adjusted p-value
log2 Fold Change
Cyp4a10
Gsta3
Cd36
Me1
Aldh1a1
Pdk4
Cyp4f15
Rimkla
Hsd17b11
Mfsd7c
Slc47a2
Fgf21
Lpin2
Nsdhl
Fam188b
6330403A02Rik
Apoa2
Slc35e3
C1qtnf7
Acot1
Doc2b
Abcc12
Edn1
Ccbe1
Hsd3b2
Ndrg1
Me3
Tia1
Mras
Pqqr7
Scn1a
Slc47a1
Cnksr2
Thpo
Il33
Traf6
Car2
Spp1
Hsph1
Il22ra2
Clec2i
Tnni3k
Dusp6
Ccl25
Crym
Cxcl13
Sell
Gab3
B
Metabolic process
Oxidation-reduction process
Transport
Long-chain fatty acid metabolic process
Fatty acid metabolic process
Glutathione metabolic process
# of genes
C
Control
Hydroxytyrosol
Relative expression
6330403a02rik
c1qtnf7
car2
ccbe1
ccl25
cd36
clec2l
cnksr2
crym
cxcl13
cyp4a10
cyp4f15
doc2b
dusp6
edn1
fgf21
fkbp5
gab3
gsta3
hsd17b
hsd3b2
hsph1
il22ra2
il33
ksr2
lpin2
mras
nrdg1
nsdhl
pqqr7
pdk4
rimkla
sell
slc35e3
slc47a2
spp1
thpo
tia1
traf6
E
Control
Hydroxytyrosol
Relative expression
aldh1a1
me1
me3
mfsd7c
nrdg1
slc47a1
D
mmu-miR-193a-5p
mmu-miR-346-5p
mmu-miR-687
mmu-miR-450b-5p
mmu-miR-1247-5p
mmu-miR-1931
mmu-miR-675-3p
mmu-miR-135a-1-3p
mmu-miR-1898
mmu-miR-23a-5p
mmu-miR-491-3p
mmu-miR-1957
mmu-miR-1954
mmu-miR-196b-3p
mmu-miR-743b-5p
mmu-miR-1894-3p
mmu-miR-1982-5p
mmu-miR-1907
mmu-miR-483-5p
mmu-miR-431-3p
mmu-miR-871-5p

gene expression in microarrays were considered as valid interactions. Target dot sizes are directly correlated with the number of interactions with the miRNA's set.

2.8. RT-qPCR validation

RT-qPCRs of selected genes to validate microarray results from intestine samples were performed in 384-well plates and gene expression was determined using the 7900HT Real-Time PCR System (Life Technologies, Spain). Reactions were performed with 5 μl of miScript SYBRGreen qPCR Master Mix (Qiagen, Madrid, Spain) following these cycling conditions: 15 min at 95°C for one cycle, then 40 cycles at 94°C for 15 s and 58°C for 30 s. The dissociation stage was analyzed at 95°C for 15 s, followed by one cycle at 60°C for 15 s and 95°C for 15 s. Gene expression analysis was carried out for 50 genes. 18S and RPLP0 were used as reference genes for normalization. Reactions were run in triplicate and relative expression of these selected genes was calculated by the comparative Ct method and presented as $2^{-\Delta\Delta Ct}$.

2.9. Validation of miRNA expression

RT-qPCRs of selected miRNAs were performed to validate qPCR array results of intestinal samples and other tissues, by miScript II RT Kit (Qiagen), following the manufacturer's protocol. Reactions were performed in 384-well plates, using SYBR green Supermix (Qiagen), and gene expression was determined using the 7900HT Fast Real-time PCR System (Applied Biosystems). Primers specific for human or mouse miRNAs were used and values were normalized using RNU1A1 and RNU6 as housekeeping small RNAs.

2.10. In vitro studies

For *in vitro* studies, human colonic adenocarcinoma cell line (Caco-2), human primary epithelial intestinal cells (InEpCells) and mouse primary organoids were used. Caco-2 cells were obtained from the American Type Culture Collection (Manassas, USA). Cells were maintained at 37°C and 5% CO_2 in DMEM medium (Lonza, Switzerland) containing 10% fetal bovine serum (HyClone, USA) and antibiotic. For differentiation, Caco-2 cells were split and seeded at a density of 5×10^5 cells/well on 24-mm-diameter polycarbonate Transwell filter inserts of 0.4 μm pore size (Costar, Madrid, Spain). Monolayers were cultured for 21 days. Differentiated Caco-2 cells were treated with HT (10 μM) or vehicle for 24 h prior to miRNAs analysis by RT-qPCR.

InEpCells were purchased from Lonza (Switzerland) and cultured in a coating of rat-tail type 1 collagen (BD Biosciences). Cells were maintained at 33°C and 5% CO_2 in SmGM-2 smooth muscle growth medium 2 (Lonza) containing SmGM-2 Bullekit (Lonza) for 7 days before experiment. InEpCells were treated with HT (10 μM) or vehicle for 24 h prior to miRNAs analysis by RT-qPCR.

For mouse primary intestinal organoids or "miniguts", mice were sacrificed and their small intestines were harvested. The intestine was flushed with ice-cold PBS and intestinal crypts isolated using IntestiCult (Stem Cell Technologies, Grenoble, France) protocol. Briefly, the small intestine was disaggregated by pipetting up and down with 15 ml of ice-cold PBS for 20 times. Tissues were rocked with 25 ml of cell dissociation reagent (Stem Cell Technologies) for 15 min at room temperature and resuspended in 10 ml PBS containing 0.1% BSA. Tissue pieces were filtered through a 70-μm cell strainer (BD Biosciences) and a crypt suspension was obtained. After centrifugation, crypt fractions were resuspended in 10 ml of cold DMEM/F12 (Lonza). Crypts were suspended in a mixture (50:50) of Matrigel (growth factor reduced phenol-red free Matrigel, Corning) and complete IntestiCult Organoid Growth Medium (Stem Cell Technologies) and gently placed in the center of each well of a prewarmed 24-well plate until the Matrigel solidified. After solidification, IntestiCult Organoid Growth Medium was added and crypts were cultured at 37°C until organoids were fully developed. Seven days after plating, organoids were treated with HT (10 μM) or vehicle for 24 h prior to miRNAs analysis by RT-qPCR.

2.11. Human studies

Samples analyzed were obtained from a previous intervention study [9]. This work is registered at ClinicalTrials.gov (identifier: NCT02273622). In brief, 21 volunteers were recruited for a randomized, cross-over, placebo-controlled and double-blind intervention study. After 1-week washout, *i.e.* olive-free diet, subjects were randomly assigned to the placebo (maltodextrin) or 25 mg/d HT (which was included in an encapsulated extract of olive mill wastewater called Hytolive) group. Subjects took the respective capsules for 1 week, then volunteers crossed over to consume the other type of capsules for 1 week. Blood samples were collected in heparinized tubes (BD Vacutainer, Franklin Lakes, NJ) at each visit, processed within 2 h after extraction and peripheral blood mononuclear cells (PBMCs) were isolated. RNAs were extracted from PBMCs and our selected miRNAs candidates were analyzed by RT-qPCR.

2.12. Statistical analysis

Normality and equality of variances of variables were tested before statistical tests were conducted. Parametric tests were applied only when both requirements were met; otherwise, nonparametric tests were employed (variables in Figs. 2, 3C and E, 4 and 6 were compared using unpaired *t* tests or Mann–Whitney tests; variables in Fig. 5 were compared by one-way ANOVA using Dunnett's test for multiple comparisons; variables in Fig. 7 were compared by two-way ANOVA followed by Sidak's test for multiple comparisons). Data are reported as mean ± SEM. The level of significance was set at $P<.05$ for all analyses. Statistical analysis was performed using IBM SPSS (version 19.0) for Windows.

3. Results

3.1. The expression of intestinal miRNAs is modulated by HT

To gain insight into novel targets of dietary polyphenols — as small-molecule modulators of miRNAs [18] — we performed an unbiased whole genome screening of miRNAs in the small intestines of mice fed with HT for a prolonged period [13]. Several intestinal miRNAs were found to be induced or repressed in response to HT feeding (Fig. 1), supporting an miRNA modulation action for this low-molecular-weight molecule.

3.2. HT supplementation has varied effects on lipid concentrations

Previous results from other laboratories [19] suggested that phenolic-enriched products could have adverse effects on lipid metabolism. In order to corroborate such data and our preliminary findings [13], we performed a second experiment with a new cohort study. For this, animals ($n=10$ per group) were fed with either chow diet or HT-enriched diet during 8 weeks. Plasma total cholesterol (Fig. 2A) concentration was not modified. However, plasma total triacylglycerol (Fig. 2B) concentrations were slightly, but significantly, increased in response to HT supplementation, whereas plasma phospholipids (Fig. 2C) were not modified. The distribution of cholesterol and triacylglycerol among the different plasma lipoproteins after supplementation or control corroborated these findings (Fig. 2D). To determine whether these changes in the plasma lipid profile increased the accumulation of lipids in the intestine, we also evaluated triacylglycerol (Fig. 2E) and cholesterol levels in this tissue (Fig. 2F). While tissue triacylglycerol levels increased, cholesterol remained unchanged. Phospholipids levels also remained constant in the intestine (Fig. 2G). To confirm these findings in another tissue, we evaluated lipid concentrations in liver samples. Similar to the intestine, triacylglycerol (Fig. 2H), but not cholesterol (Fig. 2I) or phospholipids (Fig. 2J), concentrations were altered by HT. Overall, these findings suggest that — at least in rodents — long-term HT supplementation might disturb lipid metabolism, but its physiological significance remains to be clarified.

3.3. HT-modulated intestinal genes are influenced by miRNA–gene interaction

To search for novel intestinal targets of HT, we performed a whole transcriptome expression profile (mRNA microarrays) of small intestines. A total of 674 genes were found to be differentially modulated by HT supplementation (Fig. 3A), of which 332 genes were down-regulated and 342 were up-regulated. Compared to previous adipose tissue analysis, genes including Acox2, Pibf1, Mras Asb11, Sh3gl2, Slc35f3, Fsd2, Acad10, Cyp4b1, Retsat, Pmm1, Mgst1, Gramd1a, Cblb, Apom and Vnn3 were found to be modulated, which confirms previous transcriptomic studies [13]. Functional analysis of intestinal modulated genes indicated that major biological processes modulated by HT include oxidative stress, lipid metabolism and other metabolic processes (Fig. 3B). Some of the HT-modulated miRNAs target triglyceride metabolic-related genes (Supplementary Table 1) which might be associated with increased triacylglycerol levels observed. We next chose some key genes involved in these biological processes to validate our transcriptomic data by RT-qPCR. We found that genes including Car2, Crym, Cxcl13, Dusp6, Hsph1 and Il33 were up-regulated, while Cd36, Cyp4a10, Gsta3, Hsd3b2, Paqr7, Pdk4 and Slc35e3 were down-regulated in response to HT feeding (Fig. 3C).

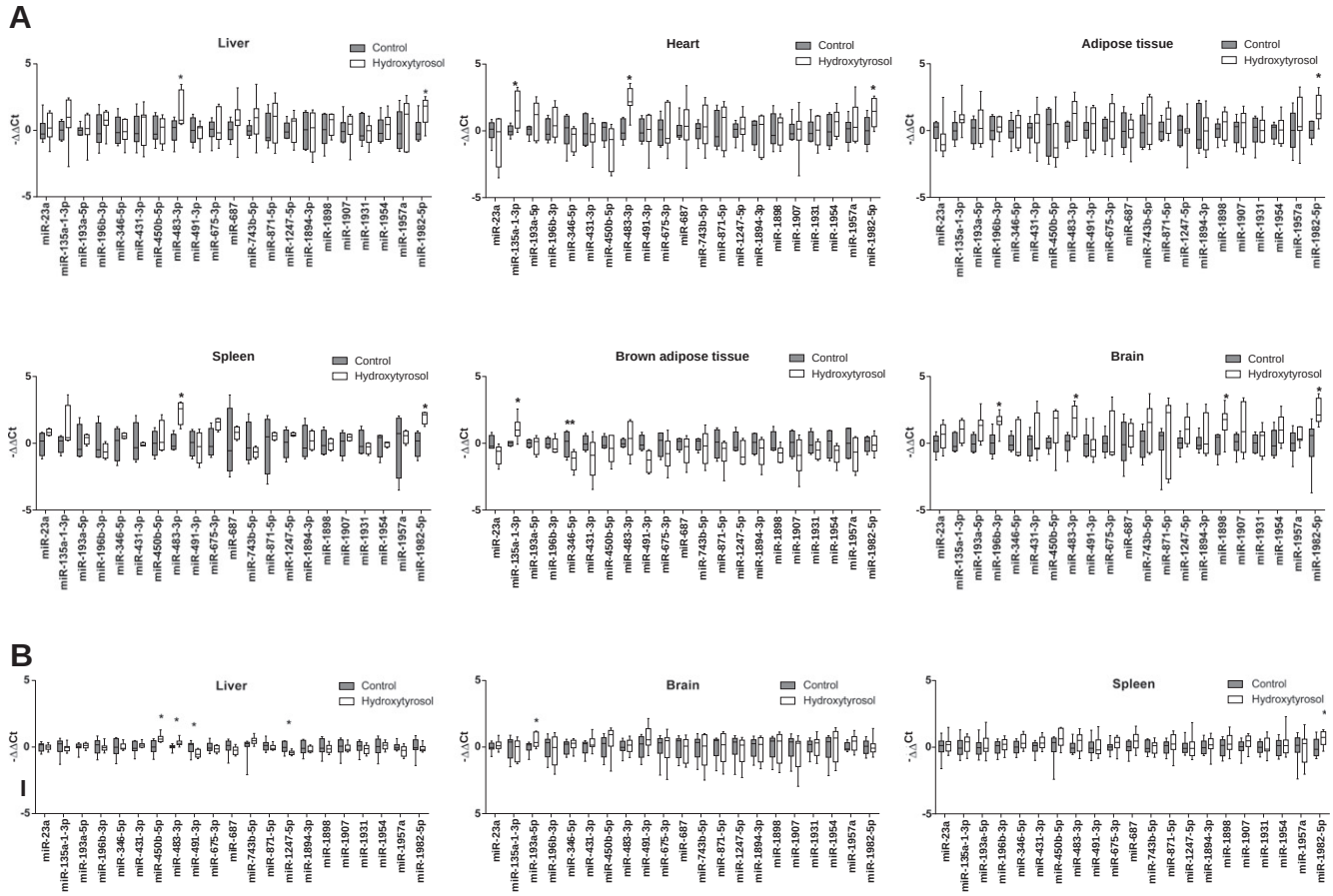


Fig. 4. HT-modulated miRNAs in different tissues. RT-qPCR analysis of selected miRNAs in different tissues from (A) first cohort and (B) second cohort study (see the materials and methods section for details). *, significantly different from controls at $P<.05$.

One of the most fascinating features of miRNA function is that they can target different genes at the same time, generally producing either target mRNA degradation or translational inhibition [20]. We thus performed an *in silico* interaction analysis (detailed above in Materials and methods) of genes likely to be modulated by combined miRNAs' expression. As one gene can be likely modulated by several miRNAs, we analyzed all putative GIs (number of HT-modulated miRNAs that can potentially target the 3´-UTR of the protein coding gene showed) (Fig. 3D). Several down-regulated genes were predicted to be targeted by more than one of these selected miRNAs. Me3 (13 GI), Mras (12 GI), Ndrg1 (12 GI), Ccbe1 (11 GI), Fam188b (11 GI), Hsd17b11 (11 GI), Lpin2 (11 GI), Pibf1 (11 GI), Tia1 (11 GI), C1qtnf7 (10 GI), Fkbp5 (10 GI), Lrrtm4 (10 GI) and Spsb4 (10 GI) are potentially targeted by more than 9 miRNAs each. Several other genes have ≤9 GIs. Validation by RT-qPCR of selected genes (Fig. 3E) confirms these findings. While we do not discard other mechanisms of genetic regulation, our findings suggest that HT-modulated miRNAs contribute to the regulation of genes involved in oxidative stress, lipid metabolism and other metabolic processes.

3.4. miRNAs modulation by HT in different tissues

A solid miRNA target of a specific molecule is likely to be modulated in different tissues after supplementation, although some exceptions may exist. In order to determine the effects of HT on other tissues, we evaluated, by RT-qPCR, the expression of selected miRNAs candidates in different tissues from the first cohort. To this end, spleen, liver, heart, white adipose tissue, brown adipose tissue and brain were evaluated for our 21 selected miRNAs candidates (Fig. 4A). Very few miRNAs were found to be modulated in these tissues in response to HT supplementation, including miR-483-3p and miR-1982-5p in the liver; miR-135a-1-3p, miR-483-3p and miR-1982-5p in the heart; miR-1982-5p in white adipose tissue; miR-483-3p and miR-1982-5p in spleen; miR-135a-1-3p, miR-346-5p and miR-491-3p in brown adipose tissue; and miR-196b-3p, miR-483-3p, miR-1898 and miR-1982-5p in brain. To confirm some of these findings, we evaluated these same miRNA candidates in liver, spleen and brain tissues in our second cohort of samples (Fig. 4B). Consistent modulation of miR-483-3p in liver and miR-1982-5p in spleen was seen in both cohorts after HT supplementation.

Except for miR-1982, for which a human homolog does not exist, the other 6 miRNAs candidates of modulation by HT (miR-135a-1-3p, miR-193a-5p, miR-196b-3p, miR-483-3p, miR-1247-5p and miR-346-5p) were selected for further characterization.

3.5. Acute ingestion of HT also modulates miRNAs' expression

To determine whether the observed effect of long-term (8 weeks) HT supplementation is also observed when it is administered acutely, we performed an acute time-course response to a single ingestion of HT. Mice ($n=9$ per group) were intragastrically administered with 15 mg of HT dissolved in water and were sacrificed at baseline (0 h), 1, 2

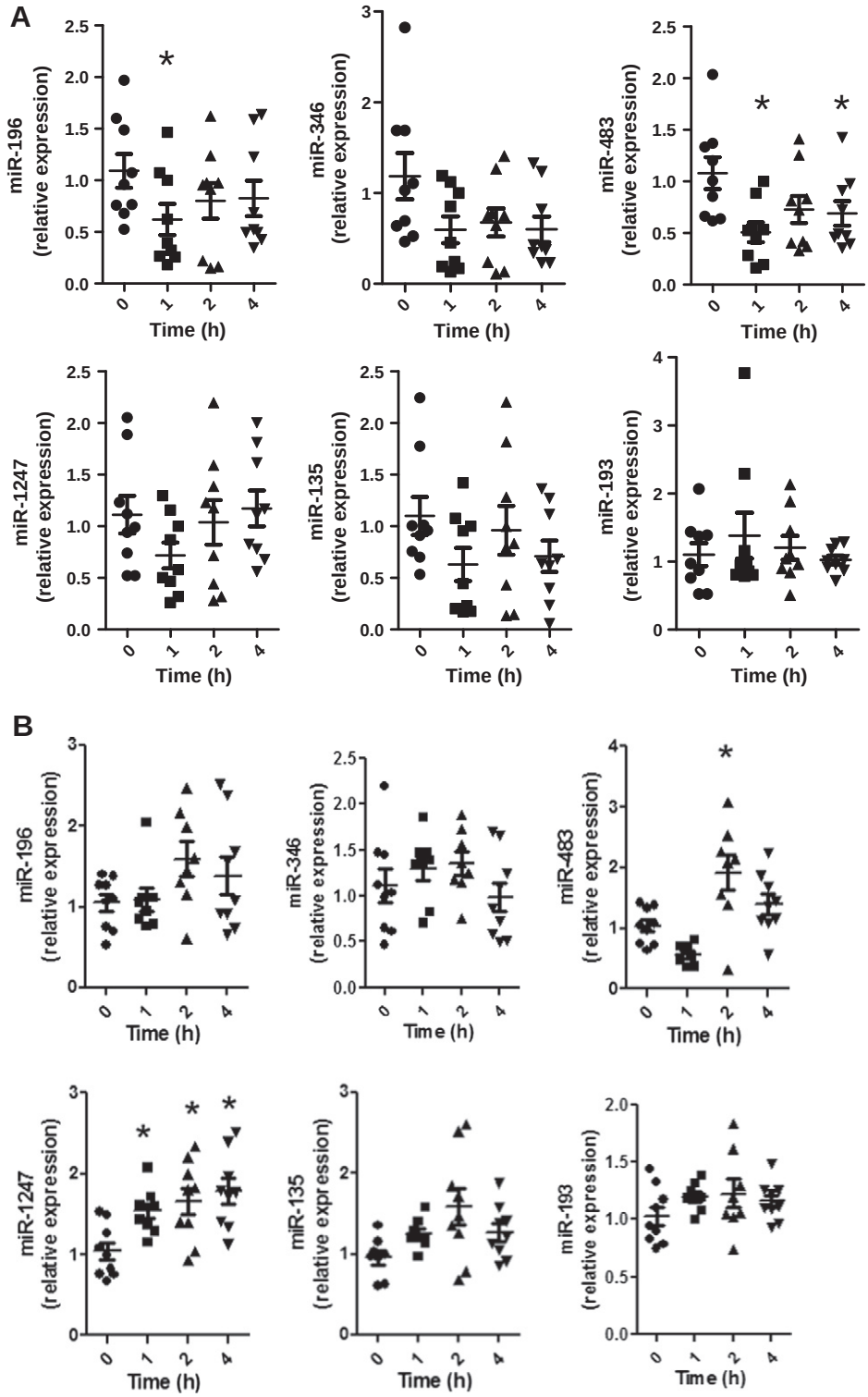


Fig. 5. Acute HT consumption modulates miRNAs expression. Time-course expression of miRNAs in response to an acute gavage of HT (15 mg per mice). RT-qPCR analysis of selected miRNAs in the (A) small intestine or (B) liver. *, significantly different from controls (time 0 h) at $P<.05$.

and 4 h after oral gavage. The six selected miRNAs were evaluated by RT-qPCR (Fig. 5A). Acute administration of HT reduced the expression of miR-196b-3p and miR-483-3p in the small intestine. To verify whether this acute administration is sufficient to modulate the expression of miRNAs in other tissues, we also evaluated the selected candidates in liver samples by RT-qPCR (Fig. 5B). In contrast to the intestine, miR-483-3p was induced in the liver. Interestingly, miR-1247-5p was also consistently induced in all time points evaluated. Taken together, these data suggest that HT could also acutely modulate the expression of certain miRNAs, and miR-483-3p, in particular, is further supported as a consistent target of modulation by this molecule.

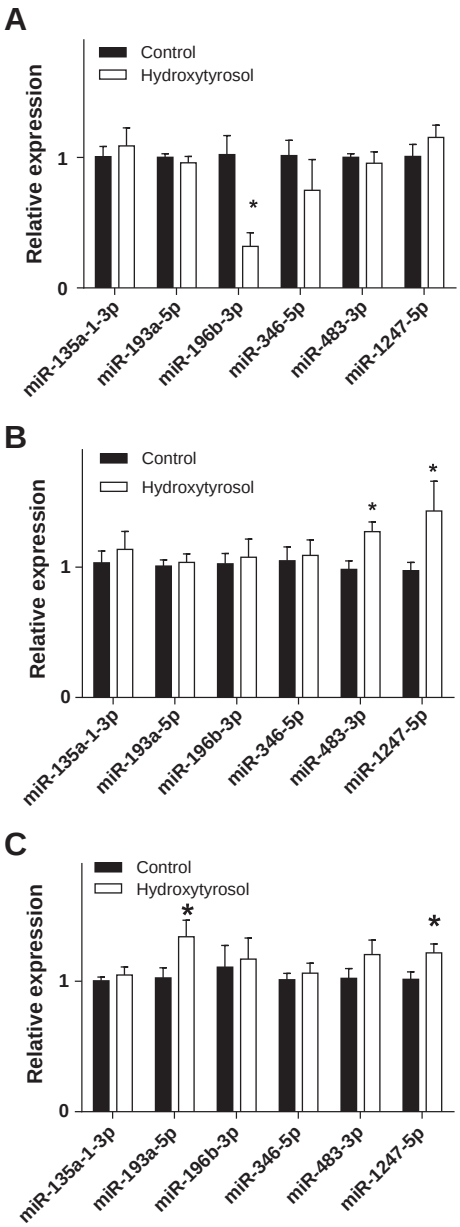


Fig. 6. HT modulates miRNAs expression in cell systems. (A) Differentiated Caco-2 cells. (B) Human primary epithelial intestinal cells. (C) Mouse small intestine organoids. Cells were exposed to HT (10 μM) or vehicle for 24 h prior to RT-qPCR analysis of selected miRNAs. *, significantly different from controls at $P<.05$.

3.6. In vitro validation of HT-modulated miRNAs

To confirm the modulation of these miRNAs by HT, we also performed some *in vitro* experiments. We exposed differentiated Caco-2 cells to HT (10 μM) and evaluated the expression of the six miRNAs candidates by RT-qPCR. The 24-h HT treatment repressed the expression of miR-196b-5p in differentiated Caco-2 cells (Fig. 6A). To further confirm these findings, human primary epithelial intestinal cells were exposed to HT (10 μM) or vehicle for 24 h and miRNAs were evaluated by RT-qPCR. miR-1247-5p and miR-483-3p were significantly induced by HT treatment (Fig. 6B). Finally, mouse small intestinal organoids or "miniguts" [21,22] were used to test the effect of HT on miRNAs' expression. In this case, miR-193a-5p and miR-1247-5p were consistently induced in miniguts by HT treatment (Fig. 6C).

3.7. miR-193a-5p is modulated by HT supplementation in healthy subjects

To evaluate whether the selected HT-modulated miRNAs observed in the animal supplementation studies were also modulated in humans, we investigated the effects of HT supplementation (25 mg/day) in a double-blind, randomized, placebo-controlled trial (NCT02273622) [9]. One-week supplementation with HT resulted in a within-group increase in the expression of miR-193a-5p in PBMCs (Fig. 7), suggesting that HT supplementation can also modulate the expression of miRNAs in humans.

4. Discussion

This study provides the first *in vivo* evidence that HT intake modulates specific miRNAs in the small intestine of mice. Some of these miRNAs are also modulated in other tissues, strengthening the causality of our findings. Notably, some of these data were confirmed in a human setting.

Our transcriptomic analysis confirms previous results from our group [13] and those of other groups [23–25] pointing towards physiological effects of HT on oxidation–reduction processes. We now provide evidence that, in the small intestine, long-term HT supplementation also influences these pathways. In addition, HT is able to modify several lipid metabolism-associated pathways. By contrast, in Caco-2 cells, HT was found to modulate pathways related to apoptosis [26]. However, in fibroblasts, it was also observed that the age-related expression of genes associated with oxidative stress was corrected by the provision of HT [27]. When we merge the results of these studies, Dusp6 [27] and Cyp1a1 [26] stand out as commonly regulated genes, confirming that HT regulates the antioxidant network at a transcriptomic level.

miRNAs are "buffers" of gene expression and probably contribute to the stronger evolutionary constraint of protein expression levels compared to mRNA levels [28]. Our GI analysis (genes likely modulated by miRNAs) suggested that certain genes might be highly influenced by miRNAs' expression, as some of them, *i.e.* Spsb4, Lrrtm4, Fkbp5, C1qtnf7, Tia1, Pibf1, Lpin2, Hsd17b11, Fam188b, Ccbe1, Ndrg1, Mras and Me3, might be tagged by more than 9 miRNAs. Biological process analysis of genes having 5 or more GIs (55 in total) also suggested that redox (GO:0055114) and transport (GO:0006810) processes are major biological pathways influenced by HT. Although the high redundancy among miRNA targets underscores the role of a specific miRNA in gene regulation, we show here that an miRNA-mediated network influences intestinal gene expression in response to HT supplementation. We, thus, suggest that the antioxidant effects observed after HT supplementation might be — at least in part — modulated by miRNA GI.

There is still limited information about the effects of HT on plasma lipids. Previous studies in mice reported contradictory results, showing both increased [19] and decreased [13] levels of plasma cholesterol. We now found that cholesterol concentrations were not modified by HT supplementation, which is also reflected by the plasma lipoprotein profile. Other studies also found no changes in plasma cholesterol after HT supplementation [24]. We also evaluated tissue cholesterol levels in both the intestine and liver and found no significant changes. However, HT supplementation tended to increase triglycerides' concentration. This was also visually clear when intestine and liver tissues were removed. No changes in phospholipid levels in plasma or tissues were observed. Cd36-deficient mice exhibit increased plasma free fatty acids and triglyceride levels [29]. We found that Cd36 was down-regulated in mice supplemented with HT for 8 weeks, which could be related to the increase in triacylglycerol levels seen. Moreover, several other intestinal-modulated miRNAs target other genes related to triacylglycerol metabolism (Supplementary

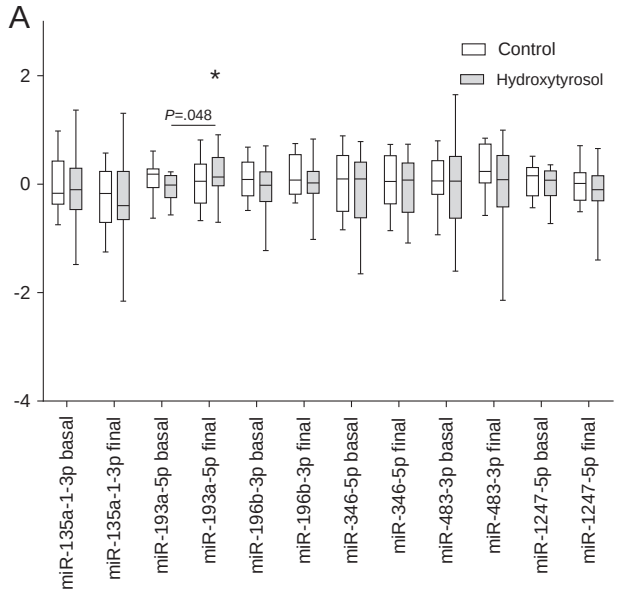


Fig. 7. HT modulates miRNAs in humans. RT-qPCR analysis of selected miRNAs analysis consuming either placebo or 25 mg HT for 1 week. *, significant difference from basal expression at $P<.05$.

Table 1). However, we do not discard any other miRNA-related or unrelated mechanism. It is important to note that lipoprotein metabolism in rodents does not perfectly match the one of humans [30] and that more appropriate humanized models for these type of studies exist [31].

We found that a selection of intestinal HT-modulated miRNAs are putative targets of modulation by HT when it is long-term supplemented (8 weeks). miR-196b-3p, miR-346-5p, miR-483-3p, miR-1247-5p, miR-135-a-5p, miR-193a-5p and miR-1982-5p were also found to be modulated in one or more of other tissues analyzed. Noteworthy consistency was seen in both cohorts in the case of miR-483-3p in liver and miR-1892-5p in spleen.

Analysis of host genes or sequences (data not shown) of these miRNAs suggested that their expression might be modulated independently and deserves further investigation. By analyzing these miRNAs, *i.e.* those only conserved in humans, we also found that an acute ingestion of HT influenced the expression of some of them either in the intestine or in the liver, namely miR-483-3p, reinforcing the notion of a direct effect. *In vitro* experiments with Caco-2 cells, human primary intestinal epithelial cells and mouse small intestine organoids confirmed some of these findings. Although the six selected miRNAs did not show the same response in every system tested, miR-483-3p and also miR-1247 were found to some extent to be consistently affected by HT exposure. One reasonable explanation for the absence of absolute consistency is that, contrary to what occurs in tissues, in the *in vitro* systems tested, many types of cells are lacking. Even in the case of mouse intestinal organoids, which contain almost all types of cells within the intestine, including absorptive enterocytes, multiple secretory cells (Paneth cells, goblet cells, enteroendocrine cells and tuft cells) and the M cells of Peyer's patches [21,32], they still lack smooth muscle cells, vasculature cells or other less abundant cell types.

In summary, some interesting notions could be identified from the series of *in vivo* and *in vitro* studies carried out. Although potential modulation of selected miRNAs by HT was not fully consistent in all models studied, miR-483-3p stood out as a robust candidate. miR-483-3p has been associated with hypertension [33], apoptosis [34] and proliferation [35]. miR-483-3p has been also associated with the capacity of adipocytes to store lipids and dysregulation of its expression could directly inhibit adipose tissue expandability and lipid storage, which in turn affect other tissues by stimulating ectopic triglyceride storage and lipotoxicity [36]. Regarding the other miRNAs and their function, miR-196b has been associated with inflammatory bowel disease [37], insulin [38] and cancer [39]; miR-346 has been implicated in rheumatoid arthritis [40], osteogenic differentiation [41] and cancer [42]; miR-1247 was implicated in cancer [43,44]; miR-135a was implicated in proliferation [45], inflammation [46] and calcification [47]; and miR-193a-5p was associated with cancer [48,49]. Although their effects in the intestine need to be evaluated, it is clear that HT could specifically modulate their expression and exert biological effects through this mechanism.

Very few studies evaluated the effect of dietary polyphenols on miRNAs modulation in humans. Resveratrol, ellagic acid or green tea catechins [3,4,50] have been previously shown to modulate miRNA expression in certain tissues. We here provide additional evidence that HT, the foremost polyphenol of extra virgin olive oil and a unique component of the Mediterranean diet, also modulates the expression of miRNAs. Among the six selected miRNAs from mice studies, miR-193a-5p was found to be induced by HT consumption in humans. In PBMCs, it was showed very recently that up-regulation of miR-193a-5p could have antiinflammatory effects by decreasing the expression of IL-12 [51].

Pharmacologically, miRNAs can be modulated by miRNA mimics, inhibitors (antisense oligonucleotides), sponges, miRNA masking or small-molecule modulators of miRNA function or expression [2]. In fact, antisense oligonucleotides [52], RG-012 (NCT02136862) or miRNA mimic (MRX34, NCT01829971) have already entered clinical trials. The search for small molecules — including those that can be found in foods — capable of modulating miRNA function and the elucidation of their mechanisms of action could represent a novel approach aimed at modulating the expression of certain miRNAs or developing drugs against specific miRNAs.

A limitation of this study is that we focused our validation experiments — both in mice tissues and human PBMCs — only within a selection of miRNAs (*i.e.* six miRNAs) that are conserved in humans. There might be other mouse or human miRNAs modulated by HT that were not evaluated in this study.

In conclusion, our data provide novel evidence to the hypothesis that dietary polyphenols can modulate miRNAs' expression. Although the precise molecular mechanism underlying the changes in miRNA expression induced by HT remains to be elucidated, our data suggest that the use of small, low-molecular-weight molecules capable of modulating miRNAs´ functions might be a viable alternative or an adjuvant to the current pharmacologic arsenal targeting endogenous miRNAs.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2016.05.009.

Author contribution

F.V. and A.D. designed the study; A.D, M.C.C., J. T.-C., J.G.Z. and E.B.-R. performed miRNAs determination and *in vitro* experiments; E.H. and D.G.-C. performed lipid determinations; A.D., M.A.L., E.I.-G., C.T.-Z. and C.C. performed animal studies; F.V., M.C.C. and J.T.-C. performed the clinical trial; J.T.-C. performed the statistical analysis; R.M. performed bioinformatics analysis; F.V., A.D., M.A.L. and E.I.-G contributed to the discussion of the manuscript. All authors have read and approved the manuscript.

The authors are not aware of any conflict of interest.

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Supplemental table 1. Hydroxytyrosol-modulated genes related to triglyceride metabolism and target of intestinal miRNAs.

Gene symbol	GO ID	GO Biological process	Fold change	adj pvalue (array)	Target of mmu-miR-:	Profile
Cd36	GO:0034197	triglyceride transport	-2,7075	0,00475066	-1931, -871-5p	miRNA up, target down
Pck1	GO:0046327	glycerol biosynthetic process from pyruvate	-2,0325	0,009130449	-431-3p, -1957, -687, -23a-5p	miRNA up, target down
Lpin2	GO:0019432	triglyceride biosynthetic process	-1,46	0,024896731	-1898, -1247-5p, -743b-5p, -1954, -1957, -483-5p, -675-3p, -871-5p, -1894-3p, -1907, 1982-5p	miRNA up, target down
Cyp2e1	GO:0006641	triglyceride metabolic process	-1,3125	0,0308357	-483-5p	miRNA up, target down
Rgn	GO:0010867	positive regulation of triglyceride biosynthetic process	-1,17	0,037066887	-346-5p, -675-3p, -1954	miRNA up, target down
Acsl1	GO:0006641	triglyceride metabolic process	-0,845	0,064781237	-431-3p, -1982-5p, -1907, -23a-5p, -687, -346-5p, -675-3p, -483-5p, -1954	miRNA up, target down
Hmgcs2	GO:0034014	response to triglyceride	-0,8775	0,066440076	-23a-5p, -483-5p, -743b-5p, -1931, -1894-3p, -1907, -1954	miRNA up, target down
Cat	GO:0006641	triglyceride metabolic process	-0,8425	0,066481224	-346-5p, -675-3p, R-1931, -1982-5p	miRNA up, target down
Angptl4	GO:0070328	triglyceride homeostasis	-0,77	0,068092023	-491-3p, -1957, -23a-5p, -871-5p	miRNA up, target down
Gpat2	GO:0019432	triglyceride biosynthetic process	-0,845	0,079641444	-743b-5p	miRNA up, target down

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Proteomic evaluation of mouse adipose tissue and liver following hydroxytyrosol supplementation

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ABSTRACT

Background and aims: Hydroxytyrosol (HT) is the primary phenolic compound of olives, virgin olive oil, and their byproducts. Proteomic analysis of metabolically active tissues helps elucidating novel mechanisms of action and potential targets in cardiometabolic disease. Thus, we aimed at determining the impact of long-term HT supplementation on the proteome of adipose and liver tissue, in mice.
Methods: C57BL/6J mice received either a control diet or a diet supplemented with nutritionally relevant doses of HT for eight weeks.
Results: HT supplementation differentially affects the adipose and liver tissues proteome, as evaluated by super-SILAC. Some oxidative stress-related proteins were modulated in both tissues, such as the multifunctional protein peroxiredoxin 1, which was consistently repressed by HT supplementation. In some cases tissue-dependent modulation was observed, as in the case of FASN.
Conclusions: This study provides interesting information on the connection between changes seen at tissue proteome level and the metabolic effects of HT. The use of this pertinent proteomics quantification approach may prove quite useful for uncovering novel potential pharmaco-nutritional targets of HT supplementation.

1. Introduction

The variegated biological activities of olive oil and its phenolic constituents are being actively investigated worldwide (Bernardini and Visioli, 2017; Hmimed et al., 2016). Indeed, the European Food Safety Authority (EFSA) granted a health claim to the cardioprotective activities (i.e. they protect low-density lipoproteins from oxidation (EFSA Panel on Dietetic Products, 2011)) of such phenolics, of which hydroxytyrosol (HT) is the foremost component. However, the precise molecular mechanisms that explain several of these potentially healthful actions have not been yet fully clarified (Bernardini and Visioli, 2017).

Mass-spectrometry (MS)–based quantitative proteomics is a powerful method that allows identifying the compilation of proteins expressed in biological systems, in addition to their interactions and modifications (Shenoy and Geiger, 2015). Metabolic labeling approaches, e.g. stable isotope labeling with amino acids in cell culture (SILAC), are being acknowledged as a robust tool in proteomic quantification, with several variations of this technique being developed in recent times (Ong, 2012). In the case of super-SILAC, unlabeled tissues from a model organism, e.g. *Mus musculus* are compared with a representative mixture of heavy-SILAC

Abbreviations: HT, hydroxytyrosol; MS, mass-spectrometry; SILAC, stable isotope labeling with amino acids in cell culture; DEG/P, differentially expressed genes/proteins; GO, gene ontology; STRING, search tool for the retrieval of interacting genes/proteins; FASN, fatty acid synthase; Car3, carbonic anhydrase 3; Prdx1, peroxiredoxin 1; FABP4, fatty acid binding protein; PBMC, peripheral blood mononuclear cells.

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labeled cell lines that act as a common internal standard (super-SILAC mix) (Chen et al., 2015). The plusses of super-SILAC makes it an ideal tool for basic and clinical research aimed at identifying protein changes and precisely quantify useful biomarkers (Shenoy and Geiger, 2015). Accurate quantification of such proteins can contribute to evaluate the biological consequences of dietary exposure to a nutrient/molecule. In this context, SILAC-MS quantitative proteomic strategies have been applied to *in vitro* studies involving exposure to (poly)phenols such as quercetin (Zhou et al., 2009) or resveratrol (Alayev et al., 2014). On the other hand, quantitative proteomics approximations applied to the impact on tissue proteome of supplementation with (poly)phenols are scant.

As data on the effects of HT on tissue proteome *in vivo* are limited, we employed MS-based quantitative proteomics, namely super-SILAC, to investigate differences in protein levels in adipose tissue and the liver, i.e. the most metabolically active tissues (Uhlen et al., 2015), of mice fed long-term with HT. We also analyzed peripheral blood mononuclear cells (PBMCs) obtained from healthy volunteers supplemented with HT for one week.

2. Material and methods

2.1. Materials

Dulbecco's Modified Eagle Medium (DMEM) R6K6 was purchased from Dundee cell (Dundee, Scotland, UK). Dialyzed fetal bovine serum (FBS), phosphate buffer saline pH 7.4 (PBS) and trypsin-EDTA was supplied by Fisher Scientific (Madrid, Spain). NaCl, NaF, ethylenediaminetetraacetic acid (EDTA), $MgCl_2$, Triton X-100, ammonium bicarbonate and formic acid were obtained from Sigma-Aldrich (Madrid, Spain). Tris and Coomassie brilliant blue were bought from Bio-Rad (Madrid, Spain). Chloroform, methanol and ethanol were purchased from Scharlab (Barcelona, Spain). Trypsin Gold-Mass Spectrometry Grade, 100 μg was purchased from Promega (Madrid, Spain). LC-MS-grade water and acetonitrile (ACN) were obtained from Fisher Scientific (Madrid, Spain).

2.2. Animals and diets

This research followed the Guide for the Care and Use of Laboratory Animals, published by the US National Research Council (Eight Edition, 2010). Animal experiments were approved by the Animal Experimentation Committee of the University Complutense of Madrid (CEA-UCM 93/2012). C57BL/6 male mice (2 months old, $n = 14$) were kept on a 12:12 light/dark cycle and acclimatized for one week before experimentation. For the dietary supplementation study, food and water were given *ad libitum*. Mice were kept for eight weeks under either a purified control diet or a purified control diet (Research Diets, Inc. New Brunswick, NJ, USA; $n = 7$ each group) with 0.03% hydroxytyrosol incorporated into the pellet (Seprox Biotech, Madrid, Spain), resulting in approximately 45 mg HT/kg bw/day (human equivalent dose (HED) of 3.6 mg HT/kg bw/day). Each diet provided 24.0%, 15.0%, and 61.0% Kcal from protein, fat and carbohydrates, respectively, and their detailed composition is described elsewhere (Giordano et al., 2014). After eight weeks of supplementation, mice were anesthetized with 0.1 mL/20 g mouse cocktail containing ketamine (100 mg/kg) and xylazine (10 mg/kg) by intraperitoneal injection, and dissected through a midline incision in the abdomen. Blood samples were collected through cardiac puncture. Phosphate-buffered saline (PBS; pH 7.4) was perfused through the portal vein for 1 min to remove any remaining blood. Tissues were quickly extracted, washed in PBS, snap-frozen in liquid nitrogen, and stored at −80 °C.

2.3. Generation of the super-SILAC mix

Mouse embryo fibroblast (3T3-L1) and mouse epithelial hepatoma cells (Hepa 1–6) were grown in "heavy" DMEM medium containing $^{13}C_6$-Lys and $^{13}C_6$-Arg and supplemented with 10% dialyzed FBS and 100 units/mL of penicillin/streptomycin. Complete incorporation of the heavy amino acids after six cell divisions was verified by MS of a protein digest (data not shown).

Completely labeled cells were washed twice with cold PBS and lysed in total lysis buffer containing 1% Triton X-100, 0.5 M NaCl, 20 mM Tris, pH 8, 10 mM EDTA, 50 mM NaF, 5 mM $MgCl_2$ and protease inhibitors. The super-SILAC mix was generated by combining equal amounts of heavy lysates from the two cell lines.

2.4. Protein isolation from adipose and liver tissues

Proteins from adipose tissues were extracted as previously described (Sajic et al., 2011). Briefly, visceral adipose tissues were homogenized with a micro pestle and sonicated with an ultrasonic probe using a buffer containing Tris 50 mM, NaCl 150 mM, EDTA 0.2 mM and protease inhibitors. The homogenate was then transferred into a glass tube and extracted with a 1:2 mixture of chloroform-methanol followed by addition of a 1:1 mixture of chloroform-water. After centrifugation at 3700×*g* during 5 min at 4 °C, proteins were found in the upper phase and the disk between phases. Finally, proteins were precipitated with acetone overnight at −20 °C and resuspended in the minimum required volume of PBS depending on the initial amount of tissue.

Ground liver tissues were homogenized in total lysis buffer with a micro pestle. The homogenate was then sonicated and incubated at 4 °C with rotatory agitation during 15 min. The sample was cleared by centrifugation at 4 °C and 15,800×*g*.

2.5. Protein identification and quantitation

Two independent super-SILAC experiments with two replicates each, were carried out for the identification of differentially expressed proteins (DEP) in the adipose and liver tissues of HT-supplemented mice in comparison to controls. The super-SILAC mix was blended with both control and treated tissues lysates independently in a 1:1 ratio based on protein concentration. 20 mg total protein extracts were separated by SDS-PAGE on 10% SDS-polyacrylamide gels. After electrophoresis, proteins were visualized by Coomassie blue staining and in-gel digested as previously described (Luque-Garcia et al., 2008).

The peptides mixtures from the different in-gel tryptic digestion fractions were loaded onto a trap column (Reprosil C18, 3 μm particle size, 0.3 × 10 mm, 120 Å pore size, SGE) and then eluted through the analytical column (Acclaim Pep Map 100, C18, 3 μm particle size, 75 μm × 150 mm, 100 Å pore size, Dionex, LC Packings) with a linear gradient of 5–95% ACN in 0.1% formic acid. Samples were delivered over 120 min by a nano-LC ultra 1D plus system (Eksigent) at a flow-rate of 200 nl/min, through the analytical column to a stainless steel nano-bore emitter (O.D. 150 μm, I.D. 30 μm Proxeon, Odense, Denmark). The resulting peptides were scanned and fragmented with an LTQ XL linear ion trap mass spectrometer (Thermo Scientific, San Jose, CA, USA) operated in data-dependent ZoomScan and MS/MS switching mode using the three most intense precursors detected in a survey scan from 400 to 1600 u (three μscans). ZoomScan mass window was set to 12 Da enabling monitoring of the entire $^{12}C/^{13}C$ isotopic envelope of most doubly and triply charged peptides. Singly charged ions were excluded for MS/MS analysis. Normalized collision energy was set to 35% and dynamic exclusion was applied during 3 min periods to avoid repetitive fragmentation of ions.

Generated raw files were converted to mgf files (Bioworks 3.3.1) for submission to the MASCOT database. A database containing the NCBInr *Mus musculus* sequences (as of September 2014, 34966 sequences) was searched using the MASCOT protein identification software (v2.3 Matrix Science). Search criteria included trypsin specificity with one missed cleavage allowed, and with methionine oxidation, $^{13}C_6$-Arg and $^{13}C_6$ −Lys as variable modifications. Minimum precursor and fragment-ion mass accuracies of 1.2 and 0.3 Da were used. To precisely identify proteins, at least one unique peptide (bold-red peptides meaning highest scoring peptide matching to protein with highest total score) with a Mascot score higher than 39 ($p < 0.05$) and a minimum total protein score of 46 ($p < 0.01$) were required. The false discovery rate (FDR) was calculated by searching the same spectra against the NCBInr *Mus musculus* decoy database. Relative quantification ratios of identified proteins based on peak area were calculated using Quixot v.1.3.26 open-source software (https://www.cnic.es/wiki/proteomica/index.php/QuiXoT). Protein ratios obtained by Quixot were verified manually for all peptides. Functional processes and subcellular localization of the proteins identified by SILAC were assigned based on the biological knowledge available in Gene Ontology (GO) annotations.

2.6. Bioinformatic analysis

The Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang da et al., 2009), a wide-ranging set of functional annotation tools, was used in order to obtain information regarding the biological processes, molecular function and cellular component associated with the list of DEP found in adipose tissue or liver, using the adjusted thresholds of P-value <0.05 and enrichment gene count >2.

To explore the interaction between the differentially expressed proteins, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (Franceschini et al., 2013) was used, setting a score of 0.4 as the reliability threshold. The STRING database provides both experimental and predicted interaction information, providing a probabilistic association confidence score. Ingenuity Pathway Analysis (IPA) was used for predicting downstream effects on biological and disease processes.

2.7. mRNA vs protein correlation analysis

We also wanted to explore eventual correlations between transcriptomic and proteomic results. Spearman correlation coefficients were used to assess how mRNA and protein expression corresponded. Transcriptome data was obtained in both adipose and liver samples from these mice has described in a previous work (Giordano et al., 2014). Briefly, Illumina MouseRef-8 v2 Expression BeadChips® with Ambion Labelling, which target approximately 25,600 well annotated Ref Seq transcripts, were used. Significant modulated genes were defined as those with an absolute fold change of >2.0 and an adjusted P value of <0.05.

2.8. Western blot

Western blot (WB) was performed using standard methods. Briefly, cells from adipose and liver tissues were lysed with RIPA buffer added with complete protease inhibitors (Sigma-Aldrich, Madrid, Spain) and quantified using the *Pierce BCA Protein Assay kit* (ThermoFisher Scientific, Madrid, Spain). Protein samples were loaded on 10% SDS-polyacrylamide gradient gels and transferred to 0.2 μm pore−size nitrocellulose membranes (Bio-Rad, Madrid, Spain). After blocking with 5% nonfat milk, membranes were incubated overnight at 4 °C with rabbit polyclonal fatty acid synthase (FASN, also known as FAS) antibody (1:1000, Cell Signaling Technology, Beverly, MA, USA), fatty acid binding protein 4 (FABP4) antibody (1:1000, Cell Signaling Technology, Beverly, MA, USA), rabbit Polyclonal carbonic anhydrase 3 (Car3) antibody (1:1000, ThermoFisher Scientific, Madrid, Spain), rabbit monoclonal peroxiredoxin 1 (Prdx1) antibody (1:1000, Cell Signaling Technology, Beverly, MA, USA) or mouse monoclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:5000, Sigma-Aldrich, Madrid, Spain). After washing with Tris-buffered saline including Tween-20, membranes were incubated with secondary antirabbit or antimouse antibodies for 1.5 h at room temperature and protein detection was carried out using the enhanced chemiluminescence system with a Clarity™ Western ECL Substrate kit (Bio-Rad, Madrid, Spain).

2.9. Human study

Samples for protein analysis by WB were obtained from a previous intervention (ClinicalTrials.gov identifier: NCT02273622) study (Crespo et al., 2015). Brief, twenty-one male volunteers were recruited for a randomized, crossover, placebo-controlled, and double blind intervention trial. After one-week of an olive-free diet, subjects were randomly assigned to either placebo (maltodextrin) or 25 mg/d HT group. Blood samples were collected and PBMCs were isolated as described previously [15]. Proteins were extracted from PBMCs and analyzed by WB as described above.

2.10. Statistical analysis

WB data results were tested for normality and equality of variances before statistical tests were carried out. Since both requirements were met in all cases unpaired t-tests were conducted.

3. Results

3.1. Super-SILAC analysis of differentially expressed proteins (DEP)

We applied super-SILAC in combination with LC-ESI−MS/MS to detect DEP in the adipose and liver tissues isolated from mice. The distribution of SILAC ratios (R_{SILAC}, referring to ratios tissue1/tissue2 for all proteins found in adipose and liver tissue) was concentrated between 1.5 and −1.5 for most proteins identified, as expected when closely analyzing related tissues in a 1:1 protein mixture. R_{SILAC} distribution is represented as $\log_{10}$ (Fig. 1).

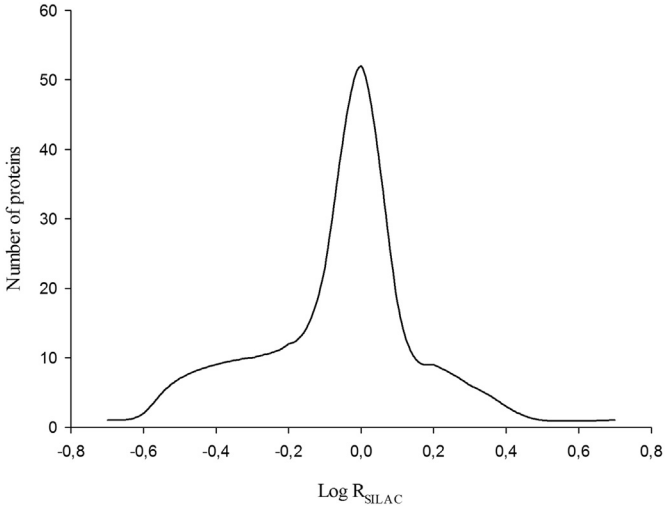

Fig. 1. Logarithm distribution of the SILAC ratio obtained.

A total of 88 proteins were identified in adipose tissue (Supplementary Table 1). Out of the proteins quantified in adipose tissue 39 were mutual to both study groups (Supplementary Table 2). Proteins were quantified with at least two unique peptides and the mean relative standard deviation (RSD) of the ratios from different peptides was lower than 20%. The overall FDR was 0.05%, being estimated by the number of hits against the reverse sequence/total hits ($p < 0.01$). Using 1.5 as the R_{SILAC} threshold ratio, 21 out of the 39 common quantified proteins were considered DEP, 12 up-regulated and 9 down-regulated (Table 1).

As regards the liver, a total of 523 proteins were identified (Supplementary Table 4). A total of 106 proteins quantified were shared by both groups (Supplementary Table 5). The higher the number of analyzed spectra, the higher is the probability of finding false positives. Since FDR is calculated according to the same MS spectra (against a decoy database with no biological meaning) and more proteins were found in liver, overall FDR used for this tissue was 0.2%. Using a threshold R_{SILAC} ratio of 1.5, 16 proteins were found to be up-regulated and 24 down-regulated out of the 106 common quantified proteins (Table 2).

3.2. Functional classifications of differentially expressed proteins (DEP)

The 21 and 40 DEP between controls and HT supplemented animals found in adipose tissue and liver, respectively, were subjected to categorical ontological analysis: Biological Process, Molecular Function and Cellular Component. In terms of biological processes, DEP found in adipose tissue were mainly associated with nervous system development, phosphorylation, and cellular protein metabolic process. Analysis of metabolic functions suggested some of these proteins are involved in RNA and protein binding. Concerning cellular component analysis most proteins were linked to the cytoplasm or with extracellular exosomes (Fig. 2A).

On the other hand, liver HT-modulated proteins, in terms of biological processes, were mainly involved in oxidative stress and oxidation-reduction responses, as well as in metabolic processes. Analysis of metabolic function established that most of these proteins play a role in oxidoreductase and lyase activity. Concerning cellular component analysis, most proteins were associated with the cytoplasmic region of the cell and with mitochondria (Fig. 2B).

Table 1
Differentially expressed proteins in the adipose tissue of mice fed a diet containing 0.03 gm% of hydroxytyrosol and controls.

Protein abbreviation	Protein full name	Mascot score	R_{SILAC}	RSD
Car3	carbonic anhydrase 3	362	3.92	10.56
Hspd1	60 kDa heat shock protein. mitochondrial	136	3.53	14.51
Cav1	caveolin-1 isoform 1	66	3.17	8.02
Hbb-b1	hemoglobin subunit beta-1	118	2.79	19.90
Trf	serotransferrin precursor	105	2.72	18.16
Fabp4	fatty acid-binding protein. adipocyte	204	2.58	18.76
Eef1a1	elongation factor 1-alpha 1	46	2.47	14.27
Hsp90ab1	heat shock protein HSP 90-beta	344	2.42	15.89
Alb	serum albumin precursor	46	2.33	17.91
Nme2	nucleoside diphosphate kinase B	93	1.83	17.31
Fasn	fatty acid synthase	70	1.71	6.97
Uba52	ubiquitin-60S ribosomal protein L40	327	1.52	17.44
Vim	vimentin	281	−1.63	18.22
Prdx1	peroxiredoxin-1	356	−1.64	18.97
Ptrf	polymerase I and transcript release factor	77	−1.75	18.12
Tpi1	triosephosphate isomerase	199	−1.89	12.41
Ywhag	14-3-3 protein gamma	80	−1.98	15.44
Ppia	peptidyl-prolyl cis-trans isomerase A	676	−2.08	17.83
Ywhaz	14-3-3 protein zeta/delta isoform 1	210	−2.08	9.94
Hist2h4	histone H4	181	−2.12	16.83
Lgals1	galectin-1	338	−3.13	3.22

Table 2
Differentially expressed proteins in the liver of mice fed a diet containing 0.03 gm% of hydroxytyrosol and controls.

Protein abbreviation	Protein full name	Mascot core	R_{SILAC}	RSD
Rpl23	60S ribosomal protein L23	124	5.38	17.29
Hbb-b1	hemoglobin subunit beta-1	1298	5.24	5.88
Aco1	cytoplasmic aconitate hydratase	438	3.22	16.45
Vcl	vinculin	402	2.63	19.75
Hpd	4-hydroxyphenylpyruvate dioxygenase	282	2.59	17.52
Ces3a	carboxylesterase 3A isoform 1 precursor	69	2.27	13.49
Hba-a2	hemoglobin alpha	745	2.16	7.13
Aldh7a1	alpha-aminoadipic semialdehyde dehydrogenase isoform b	273	1.99	14.47
Car3	carbonic anhydrase 3	1699	1.90	14.86
Gsn	gelsolin isoform 2	166	1.61	11.47
Pcx	pyruvate carboxylase. mitochondrial isoform 2	1345	1.61	17.92
Prdx2	peroxiredoxin-2	666	1.60	11.63
Cfl1	cofilin-1	1457	1.58	18.79
Hspa9	stress-70 protein. mitochondrial	647	1.57	17.47
Uba1	ubiquitin-like modifier-activating enzyme 1 isoform 1	437	1.52	17.86
Actn4	alpha-actinin-4	117	1.52	19.87
Aldh1a1	retinal dehydrogenase 1	644	−1.50	7.25
Sord	sorbitol dehydrogenase	339	−1.53	12.52
Pdia3	protein disulfide-isomerase A3 precursor	324	−1.56	13.10
Aldob	fructose-bisphosphate aldolase B	3196	−1.59	14.14
Rrbp1	ribosome-binding protein 1 isoform a	46	−1.61	14.89
P4hb	protein disulfide-isomerase precursor	385	−1.71	18.92
Aldh6a1	methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial precursor	305	−1.92	17.63
Pygl	glycogen phosphorylase. liver form	648	−1.93	19.20
Eif4a1	eukaryotic initiation factor 4A-I isoform 1	313	−1.95	15.40
Glud1	glutamate dehydrogenase 1, mitochondrial precursor	231	−1.96	12.28
Hadh	hydroxyacyl-coenzyme A dehydrogenase. mitochondrial precursor	278	−2.01	17.07
Prdx1	peroxiredoxin-1	286	−2.17	19.79
Aldoa	fructose-bisphosphate aldolase A isoform 2	1658	−2.18	9.02
Npm1	nucleophosmin isoform 1	459	−2.22	16.91
Got2	aspartate aminotransferase, mitochondrial	54	−2.22	17.19
Hadha	trifunctional enzyme subunit alpha, mitochondrial precursor	659	−2.26	17.33
Ftl1	ferritin light chain 1	434	−2.44	14.41
Aldh4a1	delta-1-pyrroline-5-carboxylate dehydrogenase. mitochondrial precursor	408	−3.20	4.38
Cat	catalase	251	−3.23	19.73
Fasn	fatty acid synthase	764	−3.69	17.58
Asl	argininosuccinate lyase	766	−3.71	11.41
Ephx2	bifunctional epoxide hydrolase 2	746	−4.31	18.49
Ass1	argininosuccinate synthase	927	−4.85	18.94
Dmgdh	dimethylglycine dehydrogenase, mitochondrial precursor	954	−5.27	18.64

3.3. Protein–protein interaction analysis and pathway annotation

A string database was used to explore possible protein–protein interactions between the DEP in both adipose tissue and liver

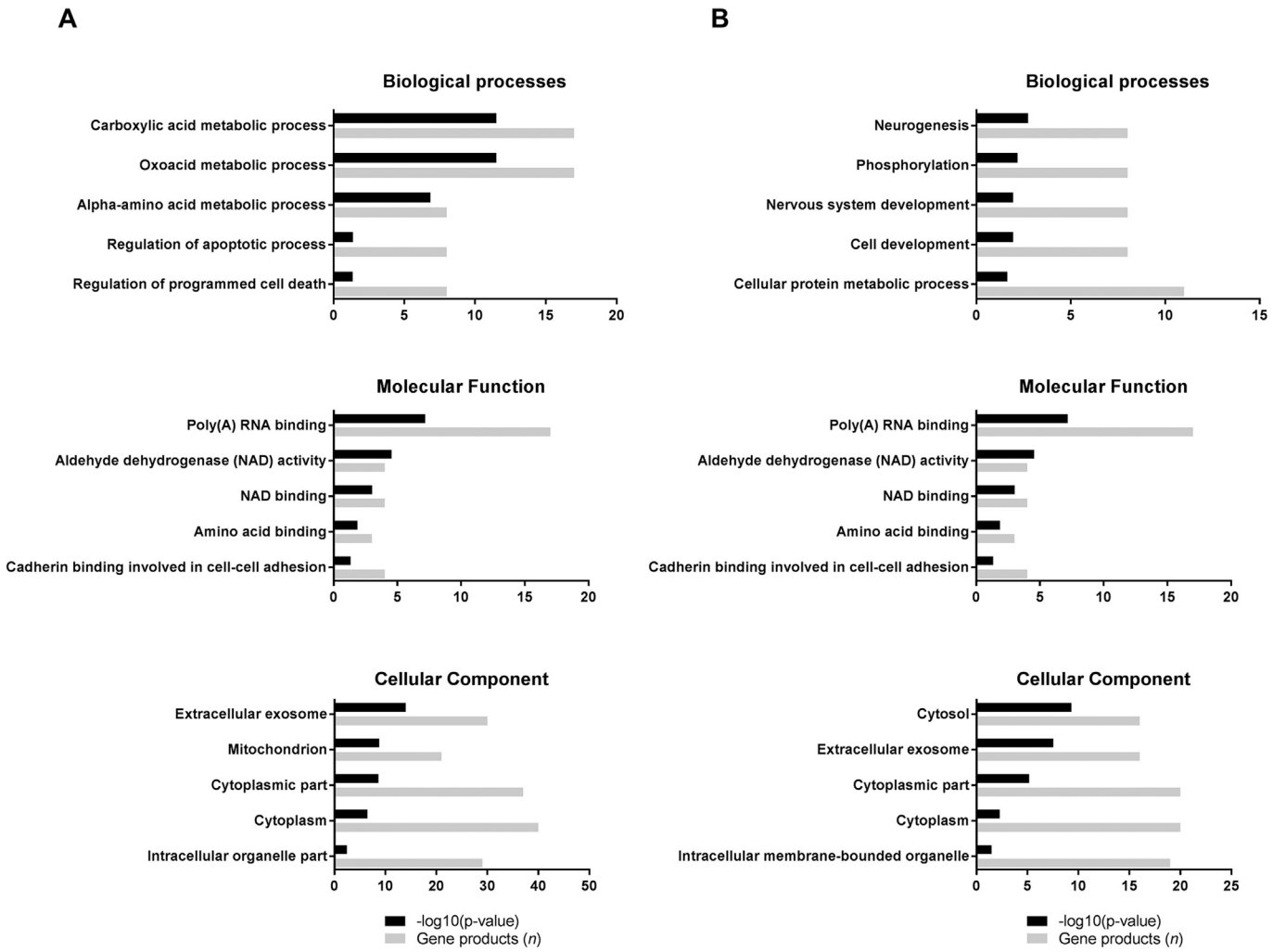

Fig. 2. Major biological processes, molecular functions, and cellular components related to the altered proteins found in mouse visceral adipose tissue (A) and liver (B).

(Fig. 3A and B, respectively). In adipose tissue, a group of 15 proteins stands out by forming a network of interactions (Cav1, Fasn, Fabp4, Eef1a, Hist2h4, Hsp90ab1, Hspd1, Nme2, Ppia, Prdx1, Ptrf, Tpi1, Uba52, Ywhaz, Ywhag), the function of which is related to protein synthesis (Mateyak and Kinzy, 2010), folding (Haase and Fitze, 2016), peroxidase activity (Rhee et al., 2012), glycolysis (Mota et al., 2009), gene expression (Kar and Chowdhury, 2015), or lipid metabolism (Capobianco et al., 2012). As for liver, a wide interaction network was found, connecting proteins related to fatty acid oxidation (Hadha), metabolism (Aco1, Got2 or Dmgdh), glycolysis (Aldoa), protein synthesis (Eif4a1), antioxidation (Prdx2) and aldehyde dehydrogenation (Aldh7a1, Aldh6a1, Aldh4a1 and Aldh1a1).

Due to the wide diversity of processes related to the DEP found in both tissues we decided to center our attention in the few DEP which could be HT targets in both tissues. Carbonic anhydrase 3 (Car3), which is associated with oxyradical scavenging, among other processes (Kharbanda et al., 2009), was the highest upregulated DEP identified in adipose tissue and was also upregulated in liver. Peroxiredoxin-1 (Prdx1), a member of antioxidant enzymes, which reduces hydrogen peroxide and alkyl hydroperoxides, was the only downregulated DEP common to both tissues. FASN, a major metabolic enzyme and a key player in fatty acid synthesis (Wu et al., 2011), was the only common DEP identified as being upregulated in one tissue and downregulated in the other. Considering the possible effects of HT on lipid metabolism (Tome-Carneiro et al., 2016), the upregulation of FABP4, which is involved in the regulation of glucose and lipid metabolism in relation to inflammatory and metabolic processes in adipocytes (Furuhashi et al., 2014), also seemed interesting.

3.4. WB validation of super-SILAC results regarding selected hydroxytyrosol-modulated proteins

Following identification by MS, presence of proteins should be validated qualitatively and/or quantitatively by other methods. WB is frequently used for this purpose, at least for proteins to which antibodies are available. Bearing this in mind, we wanted to check if the four above mentioned DEP identified, Car3, Prdx1, FASN and FABP4 (only in adipose tissue), could be reproduced by WB in a second cohort of eight-week HT-supplemented mice (Tome-Carneiro et al., 2016). Consistent corroboration of super-SILAC results in both tissues was observed regarding the downregulation of Prdx1 (Fig. 4A and B). Consistency was also found regarding FASN upregulation in visceral adipose tissue (Fig. 4A) and downregulation in liver (Fig. 4B). Car3 upregulation was not confirmed by WB in either tissue and neither was FABP4 upregulation in adipose tissue (results not shown).

Even though the aforementioned mouse tissues were the aim of this proteomic study, the consistency found for Prdx1 and FASN led

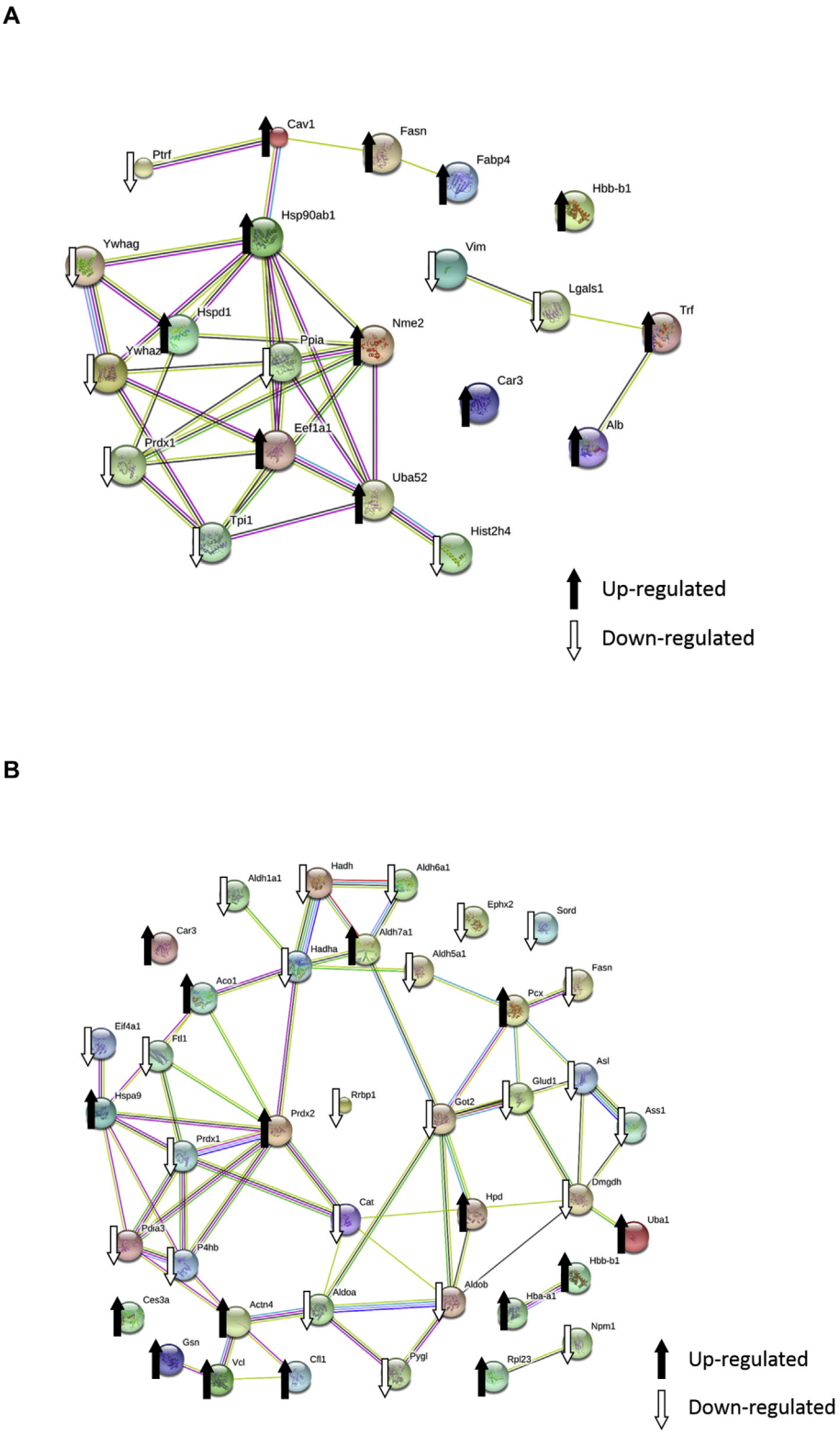

Fig. 3. Protein–protein interaction network of the differentially expressed proteins quantified in (A) adipose tissue and (B) liver, according to the Database for Annotation, Visualization and Integrated Discovery (DAVID).

us to analyze the expression of these protein in PBMCs samples isolated from volunteers who participated in a one-week HT-supplementation study (Crespo et al., 2015). Prdx1 expression was also diminished in the PBMCs of HT supplemented individuals compared to controls (Fig. 4C), whereas no difference was found for FASN (results not shown).

3.5. Proteins are poorly correlated with transcripts

To determine whether changes in protein levels resemble that of

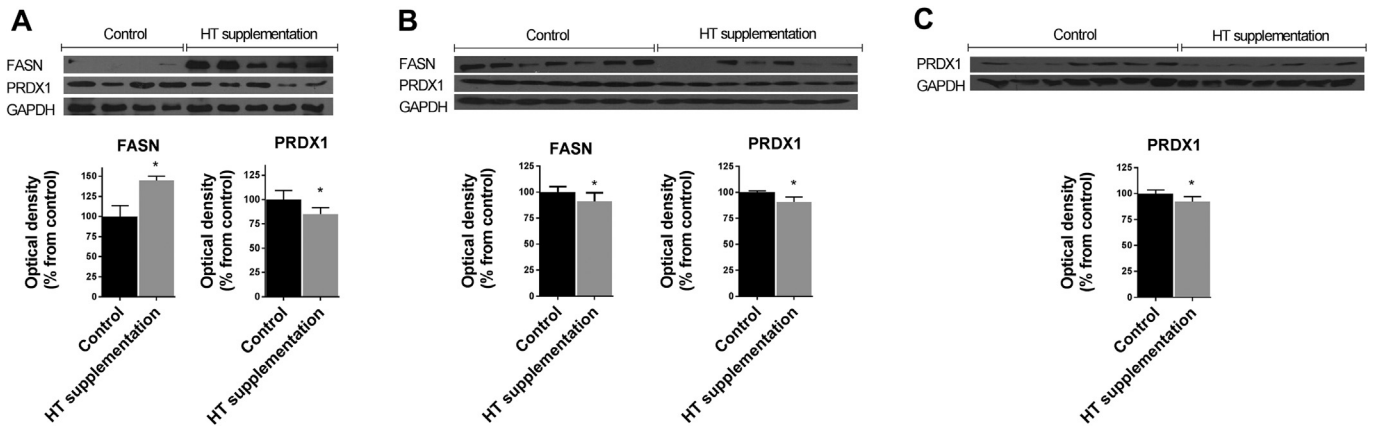

Fig. 4. Hydroxytyrosol effects on other physiological conditions. Protein analysis by Western blot of mouse visceral adipose tissue (A) and liver (B) samples of mice supplemented with a human equivalent dose of 3.6 mg hydroxytyrosol/kg bw/day, during eight weeks. (C) Blots of human PBMCs before and after a 1-week supplementation with 25 mg/d of hydroxytyrosol. Blots are representative of the whole cohort ($n \geq 6$). $^{*}P < 0.05$.

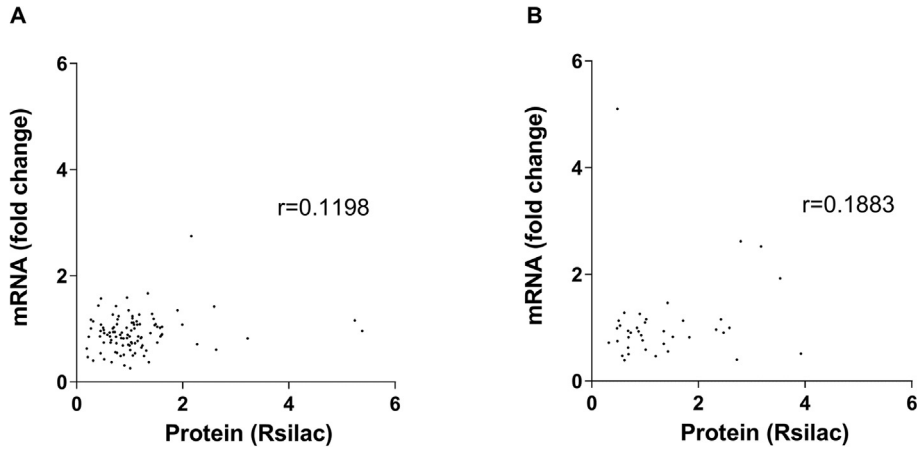

Fig. 5. Transcript-protein correlation of adipose (A) and liver (B) after hydroxytyrosol supplementation.

their transcripts, we next examined transcript-protein relationships. Commonly quantified proteins in adipose (39) and liver (106) tissues found in hydroxytyrosol-supplemented mice and their respective controls were correlated to the corresponding mRNAs by Spearman correlation coefficients. As showed in Fig. 5A and B, proteins changes were poorly correlated with transcripts.

4. Discussion

In this study, we show that long-term hydroxytyrosol supplementation has the potential to modulate protein expression in adipose tissue and the liver. Considering that these two tissues are seen as the foremost metabolically active ones (Uhlen et al., 2015), we expose that the potentially HT-modulated proteins detected here are involved in different metabolic processes including lipid metabolism and in oxidative stress response. Bearing in mind the lists of identified proteins was limited in both tissues, it is interesting that IPA examination predicted the activation of fatty acids' release and metabolism in adipose tissue, whereas lipid oxidation and lipid synthesis were predicted to be inhibited in liver. Modifications in fatty acid trafficking are likely involved in the progress of insulin resistance, and subsequent metabolic complications, and HT has been associated with an amelioration of peripheral insulin resistance *in vitro* (Crespo et al., 2017). However, the exact mechanisms through which alternate routing of fatty acids contribute to metabolic disease are yet to be ascertained (Mittendorfer, 2011). Interpretation of the existing literature regarding the effect of olive phenolics and, particularly, HT on tissue proteome is not straightforward. Treatment, species, proteomic approach, or analyzed tissues differed among investigations impeding direct comparisons. Super-SILAC in combination with LC-ESI–MS/MS analysis showed a consistent up- and downregulation for Car3 and Prdx1, respectively, while FASN was downregulated in liver and FABP4 was upregulated in adipose tissue. Loss of Car3 in preadipocytes has been shown to enhance adipogenesis (Mitterberger et al., 2012), while its reduced expression has been associated to hepatocellular carcinoma growth (Kuo et al., 2003). Hence, its upregulation seen in the liver of the animals we studied may contribute to counteract these unfavorable outcomes, although LC-ESI–MS/MS results were not confirmed by WB. Prdx1 is an antioxidant enzyme, but its physiological role in oxidization–reduction balance and in human malignances remains unclear (Jang et al., 2004). While Prdx1 knockout mice exhibited shortened life-span due to hemolytic anemia and the development of several malignant cancers, studies in humans confirmed that prdx1 is overexpressed in several types of cancer. It has also been reported that type 2 diabetes mellitus (T2DM) patients displayed higher levels of plasma Prdx1 than non-diabetics (Al-Masri et al., 2014) and that its overexpression may induce insulin resistance (Tang et al., 2015). We observed a consistent downregulation of Prdx1 in HT-supplement animals in

both liver and adipose tissue, using super-SILAC and WB, suggesting this protein may be a pharma-nutritional target of HT. Interestingly, Prdx1 was also found to be downregulated in the PBMCs of volunteers who had taken 25 mg/day of HT for one week, which reinforces the notion that the actions of HT on this protein are worth ad-hoc investigations, with possible therapeutic repercussions (Ding et al., 2017). FABP4 is involved in the regulation of glucose and lipid metabolism in relation to inflammatory and metabolic processes in target cells, e.g. participates in the transport of fatty acids and other lipids to various cellular pathways (Furuhashi et al., 2014; Hotamisligil et al., 1996; Maeda et al., 2005). Under contemporary lifestyle its overexpression may be rather disadvantageous for regulating inflammatory or metabolic homeostasis, although WB did not corroborate super-SILAC results. Finally, FASN is a multifunctional protein, which plays a critical role in a number of metabolic functions by catalyzing the terminal steps in the synthesis of long-chain saturated fatty acids (Peck and Schulze, 2016). It has been reported that excessive triacylglycerol (TG) accumulation in adipose tissue induces insulin resistance and represses adipose tissue lipogenic enzymes such as FASN (Katsurada et al., 1990). In contrast, hepatic levels of lipogenic enzymes are enhanced by the development of insulin resistance and/or T2DM (Memon et al., 1994). In this context, the upregulation found in adipose tissue and the downregulation seen in liver suggest that HT-supplementation might modulate this enzyme in a healthful way.

While the study of transcripts is helping unveil the possible biological effects of dietary supplementation with these and other phytochemicals (Giordano et al., 2014; Pereira-Caro et al., 2013), the development of large scale proteomic screening (Chick et al., 2016; Uhlen et al., 2015) is advancing our understanding of the complex regulatory variations from RNA to protein. The lack of correlation between transcripts and proteins shown here is not novel, as studies in model organisms and humans have shown that variations in mRNA and protein expression levels are often uncorrelated (Battle et al., 2015; Foss et al., 2007; Greenbaum et al., 2003; Gygi et al., 1999; Schwanhausser et al., 2011). In this respect, our previous transcriptomic (Giordano et al., 2014) analysis, and those of other groups (Granados-Principal et al., 2011; Pereira-Caro et al., 2013), did not shown any change in Car3, Prdx1, FASN or FABP4 at transcript levels (results not shown). As RNA-seq technology provides some advantages compared to microarrays, such as increased specificity and sensitivity and easier detection of rare and low-abundance transcripts (Lowe et al., 2017), gene expression analyses might be worth redoing in the future.

Yet, we are aware of the limitations regarding the results obtained and the interpretations that can be drawn from them. The amount of common quantified proteins identified in our super-SILAC proteomic approach was quite low. We are aware that several hundred or even thousand proteins have been identified in somewhat similar LTQ XL Orbitrap-MS studies (Fang et al., 2015; Meierhofer et al., 2014). The histological complexity of adipose tissue makes it technically challenging to analyze and could help explain the low protein/peptide concentration found. Nevertheless, this type of challenge is not expected for regular liver tissue samples, even though the large number of tissue-enriched gene expression found in this tissue (Uhlen et al., 2015) may contribute to the low number of commonly modulated proteins. Another contributing account concerns the restrictive criteria set for at least 2 unique peptides per protein for positive identifications. The aim of this work was to identify as much differently expressed proteins upon hydroxytyrosol administration as possible and we believe our contribution to the current knowledge on this subject, though limited, is useful. We acknowledge subsequent studies could benefit from supplementary workflow optimization. However, despite the limited number of common proteins identified, it needs to be clear that proteins in cell lines were just used as “internal standard” and the fact that most “light” peptides (coming from tissues) were coupled to “heavy” peptides (coming from SILAC-labeled cell culture lines), meaning most of the proteins found in the tissues were also in the cell lines used for the super-SILAC mix, thus confirming the suitability of this approach. In addition, several proteins reported as being modulated by HT and/or olive phenolics (Al-Masri et al., 2014; Jang et al., 2004; Kuo et al., 2003; Mitterberger et al., 2012) coincided to some extent with the ones found here. These proteins include fatty acid binding protein, glutamate dehydrogenase, ornithine carbamoyltransferase, epoxide hydrolase, nucleoside diphosphate kinase, pyruvate carboxylase mitochondrial, aconitate hydratase, 4-hydroxyphenylpyruvate dioxygenase, carboxylesterase, catalase, fructose-biphosphate aldolase, superoxide dismutase [Cu-Zn], vinculin, myosin-9, cofilin-1, vimentin, and hemoglobin subunit beta-1, among others.

Protein phosphorylation is the most common eukaryotic post-translational modification and can act as either a molecular switch or a regulator for protein functions (Vlastaridis et al., 2017). Although we did not perform phosphoproteomic enrichment, additional proteins were identified in adipose tissue and liver after expanding searches to include Y, S and T protein phosphorylation (Supplementary Tables 3 and 6, respectively).

In conclusion, the proteomic analysis approach used here represents a promising suitable tool to study the effects of food supplementation on the proteome, contributing to the potential uncovering of novel mechanisms of action and targets of micronutrients, such as HT. Our study resulted in the identification of several differently expressed proteins and pathways that justify a follow-up targeting a larger set of proteins in these pathways (for example with WB or targeted proteomics). An impact on the proteome was seen after supplementation with nutritionally relevant amounts of HT (Bernardini and Visioli, 2017; Giordano et al., 2014), especially in the case of Prdx1 and FASN, and deserves further investigation.

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Author contributions

FV and AD designed the study. J T-C, MCC, E G-C, JL L-G and AD conducted experiments. J T-C, E G-C E, and JL L-G performed data analysis. J T-C, AD, JL L-G and FV contributed to the writing of the manuscript. All authors approved the final version of the manuscript.

Conflict of interest

The authors declare that no conflict of interest exist.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fct.2017.07.009.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2017.07.009.

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Supplementary table 1. Proteins identified in adipose tissue.

GI number	Protein name	Mascot score
6671507	actin, aortic smooth muscle	264
6671509	actin, cytoplasmic 1	60
6678079	alpha-1-antitrypsin 1-1 isoform 1 precursor	79
6678085	alpha-1-antitrypsin 1-4 precursor	79
6678189	seminal vesicle secretory protein 5 precursor	96
6678483	ubiquitin-like modifier-activating enzyme 1 isoform 1	61
6678682	galectin-1	338
6679078	nucleoside diphosphate kinase B	93
6679108	nucleophosmin isoform 1	59
6679439	peptidyl-prolyl cis-trans isomerase A	676
6679567	polymerase I and transcript release factor	77
6680604	keratin, type I cytoskeletal 16	59
6680748	ATP synthase subunit alpha, mitochondrial precursor	148
6680854	caveolin-1 isoform 1	66
6680924	cofilin-1	246
6681137	acyl-CoA-binding protein isoform 2	193
6753036	aldehyde dehydrogenase, mitochondrial precursor	81
6753060	annexin A5	60
6754084	glutathione S-transferase Mu 1	61
6754254	heat shock protein HSP 90-alpha	129
6754976	peroxiredoxin-1	122
6755040	profilin-1	248
6755863	endoplasmin precursor	105
6755911	thioredoxin	89
6756041	14-3-3 protein zeta/delta isoform 1	210
6996913	annexin A2	148
7106439	tubulin beta-5 chain	253
9845265	ubiquitin-60S ribosomal protein L40	327
9910294	keratin, type II cytoskeletal 71	184
14149635	fatty acid-binding protein, adipocyte	127
14192922	actin, alpha cardiac muscle 1	60
20330802	serotransferrin precursor	105
20799907	histone H2A type 2-A	54
20911031	keratin, type II cytoskeletal 5	168
21361209	histone H4	113
21489935	keratin, type I cytoskeletal 14	65
22164776	keratin, type II cytoskeletal 79	73
29244126	histone H2A.J	276
29789317	keratin, type II cytoskeletal 75	134
31543974	14-3-3 protein beta/alpha	58
31543976	14-3-3 protein gamma	80

31560686	heat shock-related 70 kDa protein 2	116
31980648	ATP synthase subunit beta, mitochondrial precursor	167
31980922	elongation factor 1-beta	77
31981068	microsomal glutathione S-transferase 1	95
31981562	pyruvate kinase isozymes M1/M2 isoform 1	54
31981690	heat shock cognate 71 kDa protein	309
31981890	beta-2-microglobulin precursor	91
31982186	malate dehydrogenase, mitochondrial precursor	212
31982300	hemoglobin subunit beta-1	118
31982755	vimentin	377
31982861	carbonic anhydrase 3	362
40556608	heat shock protein HSP 90-beta	229
42415475	protein disulfide-isomerase precursor	73
45597447	superoxide dismutase [Cu-Zn]	1297
47059013	keratin, type II cytoskeletal 73	87
51092293	keratin, type II cytoskeletal 1b	87
52353955	D-3-phosphoglycerate dehydrogenase	356
54607171	keratin, type II cytoskeletal 6A	120
61743961	AHNAK nucleoprotein isoform 1	112
70794816	uncharacterized protein LOC433182	138
76881807	alpha-1-antitrypsin 1-2 precursor	79
84794552	phosphatidylethanolamine-binding protein 1	70
93102409	fatty acid synthase	70
112293264	protein disulfide-isomerase A3 precursor	59
112983636	keratin, type I cytoskeletal 10	206
113195684	keratin, type II cytoskeletal 6B	94
114145561	keratin, type II cytoskeletal 8	70
117553604	carboxylesterase 1D precursor	115
124487419	keratin, type II cytoskeletal 2 epidermal	48
124517663	annexin A1	75
125347376	filamin-A	65
126116585	keratin, type II cytoskeletal 1	127
145301549	hemoglobin alpha, adult chain 2	64
145580629	keratin Kb40	66
160333304	apolipoprotein A-I preproprotein	121
160358829	hemopexin precursor	46
161760667	prelamin-A/C isoform C	51
163310765	serum albumin precursor	123
164698408	perilipin-1	90
183396771	60 kDa heat shock protein, mitochondrial	136
226823220	keratin, type I cytoskeletal 15	61
226874906	14-3-3 protein epsilon	66
226958349	triosephosphate isomerase	199
227116343	platelet glycoprotein 4	94

251823978	pyruvate carboxylase, mitochondrial isoform 2	67
254540166	78 kDa glucose-regulated protein precursor	124
257153450	semenogelin I precursor	314

Supplementary Table 2. Commonly quantified proteins in the adipose tissue of mice fed a diet containing 0.03 gm% of hydroxytyrosol and controls.

Protein abbreviation	**Protein full name**	**Mascot score**	**R_{SILAC}**	**RSD**
Car3	carbonic anhydrase 3	362	3.92	10.56
Hspd1	60 kDa heat shock protein. mitochondrial	136	3.53	14.51
Cav1	caveolin-1 isoform 1	66	3.17	8.02
Hbb-b1	hemoglobin subunit beta-1	118	2.79	19.90
Trf	serotransferrin precursor	105	2.72	18.16
Fabp4	fatty acid-binding protein. adipocyte	204	2.58	18.76
Eef1a1	elongation factor 1-alpha 1	46	2.47	14.27
Hsp90ab1	heat shock protein HSP 90-beta	344	2.42	15.89
Alb	serum albumin precursor	46	2.33	17.91
Nme2	nucleoside diphosphate kinase B	93	1.83	17.31
Fasn	fatty acid synthase	70	1.71	6.97
Uba52	ubiquitin-60S ribosomal protein L40	327	1.52	17.44
Hba-a2	hemoglobin alpha. adult chain 2	64	1.43	19.52
Anxa1	annexin A1	67	1.42	6.12
Atp5b	ATP synthase subunit beta. mitochondrial precursor	167	1.35	4.15
Hspa5	78 kDa glucose-regulated protein precursor	124	1.35	17.75
Hspa8	heat shock cognate 71 kDa protein	309	1.2	17.61
Anxa5	annexin A5	60	1.02	11.09
Pfn1	profilin-1	248	1.01	16.00
Hist2h2bb	histone H2B type 2-B	40	1	7.81
Eno1b	uncharacterized protein LOC433182	138	-1.05	17.1
Serpina1e	alpha-1-antitrypsin 1-5 precursor	229	-1.08	9.70
Krt10	keratin. type I cytoskeletal 10	294	-1.11	8.62
Actb	actin. cytoplasmic 1	60	-1.14	17.74
Cfl1	cofilin-1	246	-1.18	17.58
Anxa2	annexin A2	148	-1.37	17.06
Sod1	superoxide dismutase [Cu-Zn]	1297	-1.45	15.05
Mgst1	microsomal glutathione S-transferase 1	95	-1.45	4.08
Tubb5	tubulin beta-5 chain	253	-1.47	17.80
Phgdh	D-3-phosphoglycerate dehydrogenase	356	-1.47	16.46
Vim	vimentin	281	-1.63	18.22
Prdx1	peroxiredoxin-1	356	-1.64	18.97

Ptrf	polymerase I and transcript release factor	77	-1.75	18.12
Tpi1	triosephosphate isomerase	199	-1.89	12.41
Ywhag	14-3-3 protein gamma	80	-1.98	15.44
Ppia	peptidyl-prolyl cis-trans isomerase A	676	-2.08	17.83
Ywhaz	14-3-3 protein zeta/delta isoform 1	210	-2.08	9.94
Hist2h4	histone H4	181	-2.12	16.83
Lgals1	galectin-1	338	-3.13	3.22

Supplementary table 3. Additional proteins identified in adipose tissue after expanding search to Y, S and T phosphorylation.

GI number	Protein name	Mascot score
83921595	acyl-CoA-binding protein isoform 1	62
30425250	beta-actin-like protein 2	73
6671664	calnexin precursor	51
19526922	keratin, type I cytoskeletal 25	57
85701680	keratin, type II cytoskeletal 2 oral	51
133778953	keratin, type II cytoskeletal 4	59
114326546	phosphoglycerate mutase 1	53
9256624	phosphoglycerate mutase 2	53
309267203	PREDICTED: keratin, type II cytoskeletal 78-like, partial	55
148747546	serine protease inhibitor A3K precursor	46
33563240	actin, alpha skeletal muscle	253
269914154	uncharacterized protein LOC239673	51

Supplementary table 4. Proteins identified in liver.

GI number	Protein name	Mascot score
17975508	1,4-alpha-glucan-branching enzyme	60
31543974	14-3-3 protein beta/alpha	592
226874906	14-3-3 protein epsilon	948
6756037	14-3-3 protein eta	265
31543976	14-3-3 protein gamma	644
6756039	14-3-3 protein theta	310
6756041	14-3-3 protein zeta/delta isoform 1	1270
254553340	17-beta-hydroxysteroid dehydrogenase 13 isoform 2 precursor	76
13385680	2,4-dienoyl-CoA reductase, mitochondrial precursor	171
7110703	26S protease regulatory subunit 8	64
19882201	26S proteasome non-ATPase regulatory subunit 2	46
6754724	26S proteasome non-ATPase regulatory subunit 7	66
31560355	2-hydroxyacyl-CoA lyase 1	59
19527294	3-alpha-hydroxysteroid dehydrogenase	81
61888838	3-hydroxyacyl-CoA dehydrogenase type-2	719
17921976	3-hydroxyanthranilate 3,4-dioxygenase	370
21704140	3-hydroxyisobutyrate dehydrogenase, mitochondrial precursor	293
22122625	3-hydroxyisobutyryl-CoA hydrolase, mitochondrial precursor	161
18700004	3-ketoacyl-CoA thiolase A, peroxisomal precursor	581
22122797	3-ketoacyl-CoA thiolase B, peroxisomal precursor	844
29126205	3-ketoacyl-CoA thiolase, mitochondrial	2262
13386034	40S ribosomal protein S13	77
6677799	40S ribosomal protein S15	114
158966704	40S ribosomal protein S16	163
6677801	40S ribosomal protein S17	134
6755368	40S ribosomal protein S18	113
12963511	40S ribosomal protein S19	107
46519156	40S ribosomal protein S24 isoform 1	145
28372479	40S ribosomal protein S25	179
6755372	40S ribosomal protein S3	124
254553321	40S ribosomal protein S3a	124
6677805	40S ribosomal protein S4, X isoform	186
254675270	40S ribosomal protein S5	110
6677809	40S ribosomal protein S6	216
6755376	40S ribosomal protein S7	46
33504483	40S ribosomal protein S9	120
224994260	40S ribosomal protein SA	225

37202121	4-aminobutyrate aminotransferase, mitochondrial isoform 1 precursor	109
33859486	4-hydroxyphenylpyruvate dioxygenase	282
115334671	4-trimethylaminobutyraldehyde dehydrogenase precursor	56
183396771	60 kDa heat shock protein, mitochondrial	2147
6671569	60S acidic ribosomal protein P0	276
9256519	60S acidic ribosomal protein P1	150
255003735	60S ribosomal protein L10a	70
13385408	60S ribosomal protein L11	250
160333553	60S ribosomal protein L12	154
33186863	60S ribosomal protein L13	79
13385472	60S ribosomal protein L14	59
83699424	60S ribosomal protein L18	322
58037465	60S ribosomal protein L18a	59
226958653	60S ribosomal protein L19 isoform 1	93
12584986	60S ribosomal protein L23	124
46430508	60S ribosomal protein L23a	233
18250296	60S ribosomal protein L24	306
6677777	60S ribosomal protein L26	93
255308899	60S ribosomal protein L3	55
16716589	60S ribosomal protein L31	132
13385044	60S ribosomal protein L35	61
30794450	60S ribosomal protein L4	132
84662736	60S ribosomal protein L6	49
31981515	60S ribosomal protein L7	93
7305443	60S ribosomal protein L7a	184
6755358	60S ribosomal protein L8	153
124486895	6-phosphogluconate dehydrogenase, decarboxylating	267
254540166	78 kDa glucose-regulated protein precursor	1887
171460960	abhydrolase domain-containing protein 14B	112
21450129	acetyl-CoA acetyltransferase, mitochondrial precursor	483
110625948	acetyl-Coenzyme A acetyltransferase 3	146
18700032	acidic leucine-rich nuclear phosphoprotein 32 family member B	54
18079339	aconitate hydratase, mitochondrial precursor	525
6671507	actin, aortic smooth muscle	674
6671509	actin, cytoplasmic 1	50
112363072	actin-related protein 2/3 complex subunit 2	56
9790141	actin-related protein 2/3 complex subunit 3	48
74271799	acyl-CoA dehydrogenase family member 11	76

100817933	acyl-CoA dehydrogenase family member 9, mitochondrial	47
24418933	acyl-CoA synthetase family member 2, mitochondrial precursor	75
6681137	acyl-CoA-binding protein isoform 2	231
16905127	acyl-coenzyme A synthetase ACSM1, mitochondrial	146
19527306	adenosine kinase isoform 1	112
262263372	adenosylhomocysteinase	1393
34328230	adenylate kinase 2, mitochondrial isoform b	303
157951604	adenylyl cyclase-associated protein 1	155
22094075	ADP/ATP translocase 2	206
6680720	ADP-ribosylation factor 4	183
240120054	aflatoxin B1 aldehyde reductase member 2	122
61743961	AHNAK nucleoprotein isoform 1	244
34610207	alanine--tRNA ligase, cytoplasmic	104
10946870	alcohol dehydrogenase [NADP(+)]	316
6724311	alcohol dehydrogenase 1	196
26080429	aldehyde dehydrogenase family 16 member A1	69
30520135	aldehyde dehydrogenase family 8 member A1	410
7106242	aldehyde dehydrogenase, cytosolic 1	138
6753036	aldehyde dehydrogenase, mitochondrial precursor	1630
171846276	aldo-keto reductase family 1 member C13	94
85719330	aldo-keto reductase family 1, member C12	70
160707894	aldose reductase	153
61097906	alpha-actinin-1	202
11230802	alpha-actinin-4	117
188035915	alpha-aminoadipic semialdehyde dehydrogenase isoform b	273
31980703	alpha-aminoadipic semialdehyde synthase, mitochondrial	449
46518506	alpha-methylacyl-CoA racemase	87
257196228	amine oxidase [flavin-containing] B	97
124517663	annexin A1	711
6996913	annexin A2	791
160707925	annexin A3	131
161016799	annexin A4	432
6753060	annexin A5	1265
31981302	annexin A6 isoform a	126
21313640	AP-2 complex subunit beta isoform b	70
160333304	apolipoprotein A-I preproprotein	66
163644329	apolipoprotein E precursor	60
6755004	apoptosis-inducing factor 1, mitochondrial precursor	99
7106255	arginase-1	2912

19526986	argininosuccinate lyase	766
6996911	argininosuccinate synthase	617
219275596	asparagine--tRNA ligase, cytoplasmic isoform 2	129
160298209	aspartate aminotransferase, cytoplasmic	246
6754036	aspartate aminotransferase, mitochondrial	213
6680748	ATP synthase subunit alpha, mitochondrial precursor	1753
31980648	ATP synthase subunit beta, mitochondrial precursor	3397
21313679	ATP synthase subunit d, mitochondrial	105
166851828	ATP synthase subunit delta, mitochondrial precursor	94
83715998	ATP synthase subunit e, mitochondrial	52
31980744	ATP synthase subunit g, mitochondrial	292
163838641	ATP synthase subunit gamma, mitochondrial isoform a	275
20070412	ATP synthase subunit O, mitochondrial precursor	368
313151222	ATP-citrate synthase isoform 1	80
19527256	ATP-dependent RNA helicase DDX1	105
38372907	ATP-dependent RNA helicase DDX39A	49
6753620	ATP-dependent RNA helicase DDX3X	90
31981310	B-cell receptor-associated protein 31	76
7709990	betaine--homocysteine S-methyltransferase 1	1110
21703976	bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing)	555
31982393	bifunctional epoxide hydrolase 2	746
227908823	bifunctional purine biosynthesis protein PURH	76
261878543	C-1-tetrahydrofolate synthase, cytoplasmic	262
7657583	calcium-binding mitochondrial carrier protein Aralar2 isoform 1	167
21704156	caldesmon 1	74
6671664	calnexin precursor	357
6680836	calreticulin precursor	195
124248512	carbamoyl-phosphate synthase [ammonia], mitochondrial precursor	5409
31982861	carbonic anhydrase 3	1699
117553604	carboxylesterase 1D precursor	126
257743052	carboxylesterase 3A isoform 1 precursor	69
226874914	carboxylesterase 3B isoform 1 precursor	354
157951741	catalase	786
6680888	CD63 antigen	50
15617203	chloride intracellular channel protein 1	82
13385942	citrate synthase, mitochondrial precursor	90

6680924	cofilin-1	1457
6671746	cofilin-2	193
34328108	collagen alpha-1(I) chain precursor	102
41152517	core histone macro-H2A.1 isoform 1	80
189409138	cullin-associated NEDD8-dissociated protein 1	46
22122387	cystathionine gamma-lyase	126
148747198	cysteine--tRNA ligase, cytoplasmic isoform 1	65
13385268	cytochrome b5	173
46593021	cytochrome b-c1 complex subunit 1, mitochondrial precursor	98
22267442	cytochrome b-c1 complex subunit 2, mitochondrial precursor	383
6753498	cytochrome c oxidase subunit 4 isoform 1, mitochondrial precursor	177
112181182	cytochrome c oxidase subunit 5A, mitochondrial precursor	183
34538601	cytochrome c oxidase subunit II	270
13385006	cytochrome c1, heme protein, mitochondrial	133
19526798	cytochrome P450 2A12 precursor	139
116268125	cytochrome P450 2C29 precursor	118
268607560	cytochrome P450 2C50 isoform 1 precursor	66
31981816	cytochrome P450 2D10	239
13386414	cytochrome P450 2D26	144
160948612	cytochrome P450 2D9	167
11276065	cytochrome P450 2E1	152
124001560	cytochrome P450 2F2 precursor	221
148747522	cytochrome P450, family 2, subfamily d, polypeptide 22	128
110347487	cytoplasmic aconitate hydratase	438
134288917	cytoplasmic dynein 1 heavy chain 1	91
62526118	cytoskeleton-associated protein 4	56
255069715	cytosol aminopeptidase	201
27532959	cytosolic 10-formyltetrahydrofolate dehydrogenase	121
31981273	cytosolic non-specific dipeptidase	79
52353955	D-3-phosphoglycerate dehydrogenase	442
170014720	D-beta-hydroxybutyrate dehydrogenase, mitochondrial precursor	214
6753618	D-dopachrome decarboxylase	395
7949037	delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial precursor	139
225543103	delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial precursor	408
34328485	delta-aminolevulinic acid dehydratase	95
9790219	destrin	53

7106289	dihydrofolate reductase	74
31982856	dihydrolipoyl dehydrogenase, mitochondrial precursor	138
257796245	dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	143
21313536	dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	114
21312520	dihydropteridine reductase	559
21311901	dimethylglycine dehydrogenase, mitochondrial precursor	954
56699423	E3 ubiquitin-protein ligase NEDD4	76
31542602	ELAV-like protein 1	46
227500281	electron transfer flavoprotein subunit alpha, mitochondrial	1467
38142460	electron transfer flavoprotein subunit beta	264
126032329	elongation factor 1-alpha 1	273
31980922	elongation factor 1-beta	329
110625979	elongation factor 1-gamma	150
33859482	elongation factor 2	78
6755863	endoplasmin precursor	187
31981810	enoyl-CoA delta isomerase 1, mitochondrial precursor	484
160333193	enoyl-CoA delta isomerase 2, mitochondrial isoform a	99
29789289	enoyl-CoA hydratase, mitochondrial precursor	238
12963667	epididymal secretory protein E1 precursor	49
19526926	ester hydrolase C11orf54 homolog	59
13487925	estradiol 17 beta-dehydrogenase 5	680
9789991	estradiol 17-beta-dehydrogenase 12	115
157951743	estradiol 17-beta-dehydrogenase 8	62
21450625	eukaryotic initiation factor 4A-I isoform 1	262
22203755	eukaryotic translation initiation factor 3 subunit C	106
83921618	ezrin	89
359279938	farnesyl pyrophosphate synthase isoform 1 precursor	170
93102409	fatty acid synthase	764
14149635	fatty acid-binding protein, adipocyte	244
8393343	fatty acid-binding protein, liver	2409
75677435	fatty aldehyde dehydrogenase	140
6753912	ferritin heavy chain	133
114326466	ferritin light chain 1	434
125347376	filamin-A	259
145966915	filamin-B	81
124487139	filamin-C	67

18252784	formimidoyltransferase-cyclodeaminase	367
9506589	fructose-1,6-bisphosphatase 1	921
6671539	fructose-bisphosphate aldolase A isoform 2	1658
21450291	fructose-bisphosphate aldolase B	3196
226823367	fumarate hydratase, mitochondrial precursor	205
240120112	fumarylacetoacetase	370
6678682	galectin-1	1701
329755239	gelsolin isoform 2	166
254553458	glucose-6-phosphate isomerase	180
6679465	glucosidase 2 subunit beta precursor	56
6680027	glutamate dehydrogenase 1, mitochondrial precursor	404
31982332	glutamine synthetase	95
390190196	glutaryl-CoA dehydrogenase, mitochondrial	57
84871986	glutathione peroxidase 1	199
31981724	glutathione S-transferase A3	492
21313138	glutathione S-transferase kappa 1	129
6754084	glutathione S-transferase Mu 1	808
6680121	glutathione S-transferase Mu 2	435
6754086	glutathione S-transferase Mu 5	97
6754090	glutathione S-transferase omega-1	203
10092608	glutathione S-transferase P 1	93
6679937	glyceraldehyde-3-phosphate dehydrogenase	1495
6753966	glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic	234
22122359	glycine N-acyltransferase	532
268835741	glycine N-acyltransferase-like protein	97
6754026	glycine N-methyltransferase	825
93102417	glycine--tRNA ligase	121
268836255	glycogen phosphorylase, liver form	648
17933768	glyoxylate reductase/hydroxypyruvate reductase	91
13277394	grpE protein homolog 1, mitochondrial precursor	51
23956104	GTP:AMP phosphotransferase, mitochondrial	119
6680047	guanine nucleotide-binding protein subunit beta-2-like 1	490
13386120	H/ACA ribonucleoprotein complex subunit 2	46
112293266	heat shock 70 kDa protein 4	226
31981690	heat shock cognate 71 kDa protein	1896
114145505	heat shock protein 105 kDa	84
13385998	heat shock protein 75 kDa, mitochondrial precursor	305

6754254	heat shock protein HSP 90-alpha	1041
40556608	heat shock protein HSP 90-beta	1507
145301549	hemoglobin alpha, adult chain 2	745
31982300	hemoglobin subunit beta-1	1890
6754222	heterogeneous nuclear ribonucleoprotein A/B isoform 2	78
226443091	heterogeneous nuclear ribonucleoprotein A0	52
6754220	heterogeneous nuclear ribonucleoprotein A1 isoform a	85
157277969	heterogeneous nuclear ribonucleoprotein A3 isoform c	288
19527048	heterogeneous nuclear ribonucleoprotein F	272
10946928	heterogeneous nuclear ribonucleoprotein H	114
13384620	heterogeneous nuclear ribonucleoprotein K	274
21313308	heterogeneous nuclear ribonucleoprotein M isoform a	94
160333923	heterogeneous nuclear ribonucleoprotein U	144
32880197	heterogeneous nuclear ribonucleoproteins A2/B1 isoform 2	172
8393544	heterogeneous nuclear ribonucleoproteins C1/C2 isoform 1	129
225735584	hexokinase-1 isoform HK1	59
6754208	high mobility group protein B1	71
6754152	histidine ammonia-lyase	64
33468857	histidine triad nucleotide-binding protein 1	78
31560697	histone H1.0	78
9845257	histone H1.2	163
254588110	histone H1.3	123
13430890	histone H1.4	130
21426893	histone H1.5	73
29244126	histone H2A.J	444
7949045	histone H2A.Z	165
21361209	histone H4	1121
111038118	hydroxyacyl-coenzyme A dehydrogenase, mitochondrial precursor	278
227499238	hydroxyacylglutathione hydrolase, mitochondrial isoform 1 precursor	59
171543858	hydroxymethylglutaryl-CoA lyase, mitochondrial precursor	190
88014720	importin subunit beta-1	320
6678281	indolethylamine N-methyltransferase	321
27754065	inorganic pyrophosphatase	141
18250284	isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial precursor	105

162417975	isocitrate dehydrogenase [NADP] cytoplasmic	429
225579033	isocitrate dehydrogenase [NADP], mitochondrial precursor	81
145580629	keratin Kb40	57
112983636	keratin, type I cytoskeletal 10	73
254540068	keratin, type I cytoskeletal 18	199
7106337	keratin, type I cytoskeletal 27	48
126116585	keratin, type II cytoskeletal 1	59
51092293	keratin, type II cytoskeletal 1b	60
85701680	keratin, type II cytoskeletal 2 oral	47
47059013	keratin, type II cytoskeletal 73	64
29789317	keratin, type II cytoskeletal 75	88
22164776	keratin, type II cytoskeletal 79	61
114145561	keratin, type II cytoskeletal 8	52
31982229	ketohexokinase	206
27229113	kynureninase	51
165932331	lactoylglutathione lyase	143
188219589	lamin-B1	49
19527026	leucine-rich repeat-containing protein 59	61
21450339	liver carboxylesterase B-1 precursor	108
6754524	L-lactate dehydrogenase A chain isoform 1	1053
31982520	long-chain specific acyl-CoA dehydrogenase, mitochondrial precursor	159
31560705	long-chain-fatty-acid--CoA ligase 1	391
58218988	long-chain-fatty-acid--CoA ligase 5	129
113195678	lysosome-associated membrane glycoprotein 1 precursor	51
169790977	major urinary protein 10 precursor	977
317008607	major urinary protein 14 precursor	1085
113930712	major urinary protein 2 precursor	521
59858561	major urinary protein 20 precursor	70
88196796	major urinary protein 3 precursor	104
254540027	malate dehydrogenase, cytoplasmic	430
31982186	malate dehydrogenase, mitochondrial precursor	1224
6754092	maleylacetoacetate isomerase isoform 1	340
31981106	mannosyl-oligosaccharide glucosidase	46
6680618	medium-chain specific acyl-CoA dehydrogenase, mitochondrial precursor	48
31980806	membrane-associated progesterone receptor component 1	115
19527258	methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial precursor	423
31981068	microsomal glutathione S-transferase 1	192
254540023	microsomal triglyceride transfer protein large subunit isoform 1 precursor	60

9790055	mitochondrial carrier homolog 2	54
254826790	mitochondrial dicarboxylate carrier	76
70778915	moesin	94
19526848	MOSC domain-containing protein 2, mitochondrial precursor	61
31982724	myb-binding protein 1A	165
33620739	myosin light polypeptide 6	67
33598964	myosin-10	107
241982716	myosin-11 isoform 1	312
114326446	myosin-9 isoform 1	1451
54292135	N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase isoform 1 precursor	48
13195624	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial precursor	77
58037109	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10	169
281485615	NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial	94
19745150	NADH-cytochrome b5 reductase 3	530
229892316	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial precursor	80
162139827	NADP-dependent malic enzyme isoform 1	239
163965357	nascent polypeptide-associated complex subunit alpha isoform a	221
6679891	neutral alpha-glucosidase AB	77
45476581	non-specific lipid-transfer protein	204
84875537	nucleolin	53
6679108	nucleophosmin isoform 1	459
37700232	nucleoside diphosphate kinase A	303
7657357	nucleosome assembly protein 1-like 1 isoform 2	67
8393866	ornithine aminotransferase, mitochondrial precursor	96
6679184	ornithine carbamoyltransferase, mitochondrial precursor	1858
9910482	PCTP-like protein	62
6679439	peptidyl-prolyl cis-trans isomerase A	1524
71774133	peptidyl-prolyl cis-trans isomerase B precursor	259
6754976	peroxiredoxin-1	286
148747558	peroxiredoxin-2	666
6671549	peroxiredoxin-6	1078
6753622	peroxisomal 2,4-dienoyl-CoA reductase	247
66793429	peroxisomal acyl-coenzyme A oxidase 1	200
31541815	peroxisomal bifunctional enzyme	660
165972342	peroxisomal coenzyme A diphosphatase NUDT7 isoform 1	78
227908837	peroxisomal trans-2-enoyl-CoA reductase	182

31560132	phenazine biosynthesis-like domain-containing protein 1	185
13385584	phenazine biosynthesis-like domain-containing protein 2	224
171543886	phenylalanine-4-hydroxylase	114
84794552	phosphatidylethanolamine-binding protein 1	373
7110683	phosphoenolpyruvate carboxykinase, cytosolic [GTP]	207
227330633	phosphoglucomutase-2	253
70778976	phosphoglycerate kinase 1	712
114326546	phosphoglycerate mutase 1	365
54292132	phosphoserine aminotransferase isoform 1	75
6679525	phosphotriesterase-related protein	57
165932375	plasminogen activator inhibitor 1 RNA-binding protein isoform 1	115
6754994	poly(rC)-binding protein 1	140
291327528	poly(rC)-binding protein 2 isoform 4	78
31560656	polyadenylate-binding protein 1	96
116517301	polypyrimidine tract-binding protein 1 isoform 1	91
94383782	PREDICTED: 40S ribosomal protein S2-like	91
149272413	PREDICTED: 60S ribosomal protein L23-like	124
63572172	PREDICTED: 60S ribosomal protein L27a-like	53
407262235	PREDICTED: adenosylhomocysteinase-like	59
407262010	PREDICTED: protein AHNAK2-like	112
162287370	prelamin-A/C isoform A	282
161760667	prelamin-A/C isoform C	366
13385656	probable 4-hydroxy-2-oxoglutarate aldolase, mitochondrial precursor	143
83816893	probable ATP-dependent RNA helicase DDX5	97
31542409	probable N-acetyltransferase CML2	52
6755040	profilin-1	215
6679299	prohibitin	222
126723336	prohibitin-2	118
7242171	propionyl-CoA carboxylase alpha chain, mitochondrial precursor	57
6755100	propionyl-CoA carboxylase beta chain, mitochondrial precursor	50
33563282	proteasome subunit alpha type-1	58
134031994	proteasome subunit alpha type-2	80
261824000	proteasome subunit alpha type-3	63
7106387	proteasome subunit alpha type-5	110
6755198	proteasome subunit alpha type-6	117

7106389	proteasome subunit alpha type-7	80
6755202	proteasome subunit beta type-3	72
6755204	proteasome subunit beta type-5	232
238231384	proteasome subunit beta type-6 precursor	58
9903607	protein disulfide-isomerase A3 precursor	324
112293264	protein disulfide-isomerase A4 precursor	109
58037267	protein disulfide-isomerase A6 precursor	378
42415475	protein disulfide-isomerase precursor	385
55741460	protein ETHE1, mitochondrial precursor	63
12963539	protein FAM49B	66
22324938	protein kinase C delta-binding protein	64
7305305	protein NDRG2 isoform 1	53
6679066	protein NipSnap homolog 1	105
21886811	protein S100-A11	252
7305395	purine nucleoside phosphorylase	162
14861844	putative ATP-dependent RNA helicase Pl10	56
31560090	pyrroline-5-carboxylate reductase 1, mitochondrial	57
26006861	pyruvate carboxylase, mitochondrial isoform 2	1345
6679261	pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial precursor	50
18152793	pyruvate dehydrogenase E1 component subunit beta, mitochondrial precursor	210
31981562	pyruvate kinase isozymes M1/M2 isoform 1	91
359807367	pyruvate kinase isozymes M1/M2 isoform 2	468
153792131	pyruvate kinase isozymes R/L isoform 1	83
33859530	quinone oxidoreductase	261
116089273	rab GDP dissociation inhibitor beta	292
157277948	radixin isoform a	120
153792001	ras GTPase-activating-like protein IQGAP1	115
7305075	ras-related C3 botulinum toxin substrate 1 precursor	55
30841008	ras-related protein Rab-1A	54
6679587	ras-related protein Rab-2A	118
148747526	ras-related protein Rab-7a	248
6677739	regucalcin	963
85861182	retinal dehydrogenase 1	644
6677697	retinol dehydrogenase 16	77
8567342	retinol dehydrogenase 7 precursor	92
31982030	rho GDP-dissociation inhibitor 1	191
40807498	ribonuclease UK114	466
124486712	ribosome-binding protein 1 isoform a	46

19526790	S-adenosylmethionine synthase isoform type-1	200
20149748	sarcosine dehydrogenase, mitochondrial	849
21362309	SEC14-like protein 2	77
22164798	selenium-binding protein 1	1208
9507079	selenium-binding protein 2	1647
160333789	sepiapterin reductase	154
6754816	septin-7 isoform 1	63
28173550	serine hydroxymethyltransferase, cytosolic	51
6755478	serine/arginine-rich splicing factor 2	71
8394027	serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	52
111038130	serine--pyruvate aminotransferase, mitochondrial	154
20330802	serotransferrin precursor	61
161353502	serpin H1 precursor	138
163310765	serum albumin precursor	319
31982522	short-chain specific acyl-CoA dehydrogenase, mitochondrial precursor	193
15147224	sideroflexin-1	87
45544618	S-methyl-5~-thioadenosine phosphorylase	137
23943838	solute carrier family 25, member 1	46
22128627	sorbitol dehydrogenase	339
77404392	staphylococcal nuclease domain-containing protein 1	221
12963591	stress-70 protein, mitochondrial	647
162461907	stress-induced-phosphoprotein 1	150
34328286	succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial precursor	113
255958286	succinyl-CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial precursor	77
46849708	succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial precursor	66
165972309	succinyl-CoA ligase [GDP-forming] subunit beta, mitochondrial precursor	133
18266680	succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial precursor	174
28202011	sulfotransferase-like protein 1	396
7709986	SUMO-activating enzyme subunit 2	66
45597447	superoxide dismutase [Cu-Zn]	626
31980762	superoxide dismutase [Mn], mitochondrial precursor	104
33859662	synaptic vesicle membrane protein VAT-1 homolog	66
227116327	talin-1	225
126521835	T-complex protein 1 subunit beta	340

6753322	T-complex protein 1 subunit delta	155
6671702	T-complex protein 1 subunit epsilon	51
238814391	T-complex protein 1 subunit eta	129
6753320	T-complex protein 1 subunit gamma	149
126723461	T-complex protein 1 subunit theta	119
6753324	thioredoxin	321
6755911	thiosulfate sulfurtransferase	273
6755763	threonine--tRNA ligase, cytoplasmic	55
27229277	trans-1,2-dihydrobenzene-1,2-diol dehydrogenase	57
30519911	transgelin-2	497
225543319	transitional endoplasmic reticulum ATPase	1080
6678359	transketolase	359
6678437	translationally-controlled tumor protein	144
165377206	translocon-associated protein subunit alpha precursor	56
21312062	transmembrane emp24 domain-containing protein 10 precursor	168
33859811	trifunctional enzyme subunit alpha, mitochondrial precursor	904
21704100	trifunctional enzyme subunit beta, mitochondrial precursor	135
226958349	triosephosphate isomerase	1217
256000788	tropomyosin alpha-1 chain isoform 6	66
47894398	tropomyosin alpha-4 chain	216
71043961	trypsinogen 7 precursor	73
6755901	tubulin alpha-1A chain	988
6678469	tubulin alpha-1C chain	1223
6678467	tubulin alpha-4A chain	782
21746161	tubulin beta-2B chain	1317
22165384	tubulin beta-4B chain	1404
7106439	tubulin beta-5 chain	1484
18017605	ubiquitin-conjugating enzyme E2 N	68
6678483	ubiquitin-like modifier-activating enzyme 1 isoform 1	437
284413688	UDP glucuronosyltransferase 1 family, polypeptide A6B precursor	153
6678499	UDP-glucose 6-dehydrogenase	161
145699099	UDP-glucuronosyltransferase 1-1 precursor	607
145864463	UDP-glucuronosyltransferase 1-9 precursor	483
306518591	UDP-glucuronosyltransferase 2B17 precursor	323
22779901	UDP-glucuronosyltransferase 2B4 precursor	151
70794816	uncharacterized protein LOC433182	2095
6678509	uricase	261

172072677	urocanate hydratase	47
21314832	UTP--glucose-1-phosphate uridylyltransferase	151
9790285	vacuolar protein sorting-associated protein 29	89
21624609	valacyclovir hydrolase precursor	196
113374154	very long-chain acyl-CoA synthetase	61
23956084	very long-chain specific acyl-CoA dehydrogenase, mitochondrial precursor	144
31982755	vimentin	1406
31543942	vinculin	402
6755963	voltage-dependent anion-selective channel protein 1	204
6755965	voltage-dependent anion-selective channel protein 2	107
45504359	V-type proton ATPase subunit E 1	49
77682555	xanthine dehydrogenase/oxidase	57

Supplementary Table 5. Commonly quantified proteins in the liver of mice fed a diet containing 0.03 gm% of hydroxytyrosol and controls.

Protein abbreviation	**Protein full name**	**Mascot score**	**R_{SILAC}**	**RSD**
Rpl23	60S ribosomal protein L23	124	5.38	17.29
Hbb-bt	hemoglobin subunit beta-1	1298	5.24	5.88
Aco1	cytoplasmic aconitate hydratase	438	3.22	16.45
Vcl	vinculin	402	2.63	19.75
Hpd	4-hydroxyphenylpyruvate dioxygenase	282	2.59	17.52
Ces3a	carboxylesterase 3A isoform 1 precursor	69	2.27	13.49
Hba-a2	hemoglobin alpha	745	2.16	7.13
Aldh7a1	alpha-aminoadipic semialdehyde dehydrogenase isoform b	273	1.99	14.47
Car3	carbonic anhydrase 3	1699	1.90	14.86
Gsn	gelsolin isoform 2	166	1.61	11.47
Pcx	pyruvate carboxylase, mitochondrial isoform 2	1345	1.61	17.92
Prdx2	peroxiredoxin-2	666	1.60	11.63
Cfl1	cofilin-1	1457	1.58	18.79
Hspa9	stress-70 protein, mitochondrial	647	1.57	17.47
Uba1	ubiquitin-like modifier-activating enzyme 1 isoform 1	437	1.52	17.86
Actn4	alpha-actinin-4	117	1.52	19.87
Anxa1	annexin A1	711	1.49	17.90
Cps1	carbamoyl-phosphate synthase [ammonia]	47	1.47	18.72
Sardh	sarcosine dehydrogenase, mitochondrial	849	1.45	19.93

Acta2	actin, aortic smooth muscle	674	1.42	8.45
Actn1	alpha-actinin-1	202	1.39	19.73
Aldh2	aldehyde dehydrogenase, mitochondrial precursor	1630	1.36	18.22
Otc	ornithine carbamoyltransferase, mitochondrial precursor	1858	1.34	16.10
Hsp90b1	endoplasmin precursor	177	1.31	19.77
Glo1	lactoylglutathione lyase	143	1.27	12.34
Cs	citrate synthase, mitochondrial precursor	90	1.24	15.15
Flna	filamin-A	259	1.22	16.65
Ppib	peptidyl-prolyl cis-trans isomerase B precursor	259	1.22	18.78
Vim	vimentin	1406	1.22	16.88
Rpl4	60S ribosomal protein L4	132	1.21	8.05
Hsp90ab1	heat shock protein HSP 90-beta	1507	1.19	18.09
Tkt	transketolase	359	1.18	9.37
Eef2	elongation factor 2	82	1.16	19.32
Atp5a1	ATP synthase subunit alpha, mitochondrial precursor	1753	1.15	19.37
Slc25a5	ADP/ATP translocase 2	135	1.14	19.48
Vcp	transitional endoplasmic reticulum ATPase	1080	1.13	19.30
Ncl	nucleolin	575	1.10	11.61
Alb	serum albumin precursor	37.9	1.10	19.16
Rps9	40S ribosomal protein S9	120	1.09	8.70
Hspa5	78 kDa glucose-regulated protein precursor	1187	1.08	19.86
Pfn1	profilin-1	215	1.07	17.72
Rps3a1	40S ribosomal protein S3a	63	1.06	14.61
Phgdh	D-3-phosphoglycerate dehydrogenase	442	1.05	12.75
Hsp90aa1	heat shock protein HSP 90-alpha	1041	1.05	16.61
Rpl23a	60S ribosomal protein L23a	233	1.05	13.44
Rpl11	60S ribosomal protein L11	189	1.04	19.62
Acaa2	3-ketoacyl-CoA thiolase, mitochondrial	1139	1.04	17.42
Rpl18a	60S ribosomal protein L18a	59	1.04	17.79
Idh1	isocitrate dehydrogenase [NADP] cytoplasmic	401	1.03	17.71
Nme2	nucleoside diphosphate kinase B	404	1.03	19.52
Atp5b	ATP synthase subunit beta, mitochondrial precursor	3397	1.01	19.80
Eef1a1	elongation factor 1-alpha 1	93	1.01	19.26
Sod1	superoxide dismutase [Cu-Zn]	626	1.00	19.57
Eno1b	uncharacterized protein LOC433182	2095	1.00	18.29
Bhmt	betaine--homocysteine S-methyltransferase 1	1110	-1.02	15.64
Hspa8	heat shock cognate 71 kDa protein	1896	-1.03	19.92
Anxa5	annexin A5	1265	-1.04	18.71
Ahcy	adenosylhomocysteinase	1393	-1.05	18.32

Rpl12	60S ribosomal protein L12	154	-1.05	16.88
Ldha	L-lactate dehydrogenase A chain isoform 1	4053	-1.06	19.93
Acaa1b	3-ketoacyl-CoA thiolase B. peroxisomal precursor	844	-1.11	14.34
Hadhb	trifunctional enzyme subunit beta, mitochondrial precursor	135	-1.12	17.24
Actb	actin. cytoplasmic 1	379	-1.13	19.09
Hist2h4	histone H4	1121	-1.15	19.97
Mdh2	malate dehydrogenase, mitochondrial precursor	1170	-1.17	15.22
Acsl1	long-chain-fatty-acid--CoA ligase 1	391	-1.19	12.45
Hspd1	60 kDa heat shock protein, mitochondrial	2147	-1.21	19.80
Canx	calnexin precursor	357	-1.22	18.25
Mdh1	malate dehydrogenase. cytoplasmic	430	-1.27	8.54
Myh9	myosin-9 isoform 1	1451	-1.30	18.91
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	1495	-1.31	19.42
Pgk1	phosphoglycerate kinase 1	712	-1.31	19.55
Ywhaz	14-3-3 protein zeta/delta isoform 1	1270	-1.33	17.46
Arg1	arginase-1	127	-1.35	16.76
Uqcrc2	cytochrome b-c1 complex subunit 2, mitochondrial precursor	383	-1.35	16.06
Rps10	40S ribosomal protein S10	62	-1.37	17.46
Tubb5	tubulin beta-5 chain	1484	-1.38	19.55
Rpl10a	60S ribosomal protein L10a	70	-1.40	17.51
Aco2	aconitate hydratase, mitochondrial precursor	525	-1.42	18.74
Rpl24	60S ribosomal protein L24	306	-1.45	16.76
Acads	short-chain specific acyl-CoA dehydrogenase, mitochondrial precursor	242	-1.46	18.75
Ppia	peptidyl-prolyl cis-trans isomerase A	1524	-1.47	18.28
Aldh1a1	retinal dehydrogenase 1	644	-1.50	7.25
Sord	sorbitol dehydrogenase	339	-1.53	12.52
Pdia3	protein disulfide-isomerase A3 precursor	324	-1.56	13.10
Aldob	fructose-bisphosphate aldolase B	3196	-1.59	14.14
Rrbp1	ribosome-binding protein 1 isoform a	46	-1.61	14.89
P4hb	protein disulfide-isomerase precursor	385	-1.71	18.92
Aldh6a1	methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial precursor	305	-1.92	17.63
Pygl	glycogen phosphorylase. liver form	648	-1.93	19.20
Eif4a1	eukaryotic initiation factor 4A-I isoform 1	313	-1.95	15.40
Glud1	glutamate dehydrogenase 1, mitochondrial precursor	231	-1.96	12.28

Hadh	hydroxyacyl-coenzyme A dehydrogenase, mitochondrial precursor	278	-2.01	17.07
Prdx1	peroxiredoxin-1	286	-2.17	19.79
Aldoa	fructose-bisphosphate aldolase A isoform 2	1658	-2.18	9.02
Npm1	nucleophosmin isoform 1	459	-2.22	16.91
Got2	aspartate aminotransferase, mitochondrial	54	-2.22	17.19
Hadha	trifunctional enzyme subunit alpha, mitochondrial precursor	659	-2.26	17.33
Ftl1	ferritin light chain 1	434	-2.44	14.41
Aldh4a1	delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial precursor	408	-3.20	4.38
Rpl23	catalase	251	-3.23	19.73
Hbb-bt	fatty acid synthase	764	-3.69	17.58
Aco1	argininosuccinate lyase	766	-3.71	11.41
Vcl	bifunctional epoxide hydrolase 2	746	-4.31	18.49
Hpd	argininosuccinate synthase	927	-4.85	18.94
Ces3a	dimethylglycine dehydrogenase, mitochondrial precursor	954	-5.27	18.64

Supplementary table 6. Additional proteins identified in liver after expanding search to include Y, S and T protein phosphorylation.

GI number	Protein name	Mascot score
6677799	40S ribosomal protein S15	114
219276601	asparagine--tRNA ligase, cytoplasmic isoform 1	116
82617575	bifunctional glutamate/proline--tRNA ligase	711
6753284	caspase-3	325
148747410	coatomer subunit delta	76
339895744	collagen alpha-3(VI) chain isoform 1 precursor	96
126518317	complement C3 precursor	49
113680661	corticosteroid 11-beta-dehydrogenase isozyme 1	77
157154304	cytochrome P450 2D11	184
6681273	elongation factor 1-alpha 2	78
54287684	elongation factor 1-delta isoform b	138
60687506	fructose-bisphosphate aldolase C	103
32401425	glutathione S-transferase P 2	82
21450105	glutathione S-transferase P-like	149
183980004	heterogeneous nuclear ribonucleoprotein L	64
31560689	hydroxymethylglutaryl-CoA synthase, mitochondrial precursor	628

9789985	isovaleryl-CoA dehydrogenase, mitochondrial precursor	54
21489935	keratin, type I cytoskeletal 14	47
226823220	keratin, type I cytoskeletal 15	47
6680604	keratin, type I cytoskeletal 16	48
19526922	keratin, type I cytoskeletal 25	47
283436218	mitochondrial 10-formyltetrahydrofolate dehydrogenase	74
124486921	MOSC domain-containing protein 1, mitochondrial	59
19526852	nicotinate-nucleotide pyrophosphorylase [carboxylating]	59
6679078	nucleoside diphosphate kinase B	358
21313144	obg-like ATPase 1 isoform a	62
7948999	peroxiredoxin-4 precursor	81
309266416	PREDICTED: 40S ribosomal protein S15-like isoform 1	50
309266590	PREDICTED: 60S ribosomal protein L10-like	85
309267049	PREDICTED: 60S ribosomal protein L17-like	81
149272413	PREDICTED: 60S ribosomal protein L23a-like	166
51712358	PREDICTED: eukaryotic initiation factor 4A-III-like	78
51765047	PREDICTED: tubulin alpha-1C chain isoform 1	856
407263446	PREDICTED: uncharacterized protein LOC667284	53
40068493	probable ATP-dependent RNA helicase DDX17 isoform 1	50
161484668	serine/threonine-protein phosphatase PP1-beta catalytic subunit	56
117938332	spectrin beta chain, non-erythrocytic 1 isoform 1	85
34740335	tubulin alpha-1B chain	387
27370342	UDP glucuronosyltransferase 2 family, polypeptide B35 precursor	143
20911031	keratin, type II cytoskeletal 5	70
113195684	keratin, type II cytoskeletal 6B	58
9910294	keratin, type II cytoskeletal 71	64
283436180	heterogeneous nuclear ribonucleoproteins C1/C2 isoform 3	126
7305229	L-lactate dehydrogenase C chain	68

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Research Communication

Hydroxytyrosol restores proper insulin signaling in an astrocytic model of Alzheimer's disease

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Abstract

Recent epidemiological evidence demonstrated that diabetes is a risk factor for AD onset and development. Indeed, meta-analyses of longitudinal epidemiologic studies show that diabetes increases AD risk by 50–100%, being insulin resistance (IR) the main binding link between diabetes and AD. Astrocytes are the foremost cerebral macroglial cells and are responsible for converting glucose into lactate and transfer it to neurons that use it as fuel, but Aβ(1-42) impairs insulin signaling and glycogen storage. Recent prospective studies showed that the Mediterranean diet is associated with lower incidence of AD. We hypothesized that hydroxytyrosol (HT, the preeminent polyphenol of olives and olive oil) could exert beneficial effects on IR associated with AD and investigated it mechanisms of action in an astrocytic model of AD. The astrocytic cell line C6 was exposed to Aβ(25-35) and co-incubated with HT for different periods. After treatment with Aβ(25-35), astrocytes' viability was significantly decreased as compared with controls; however, both pre- and post-treatment with HT prevented this effect. Mechanistically, we found that the preventive role of HT on Aβ(25-35)- induced cytotoxicity in astrocytes is moderated by an increased HT-induced activation of Akt, which is mediated by the insulin signaling pathway. In addition, we report that HT prevented the pronounced activation of mTOR, thereby restoring proper insulin signaling. In conclusion, we demonstrate that HT protects Aβ(25-35)-treated astrocytes by improving insulin sensitivity and restoring proper insulin-signaling. These data provide some mechanistic insight on the observed inverse association between olive oil consumption and prevalence of cognitive impairment. © 2017 BioFactors, 43(4):540–548, 2017

Keywords: *Alzheimer's disease; hydroxytyrosol; Mediterranean diet; insulin resistance; astrocytes*

1. Introduction

Alzheimer's disease (AD) is an irreversible neurodegenerative disorder, characterized by progressive memory deficit and neuronal loss, whose prevalence is increasing [1,2]. Neuropathologically, AD is characterized by the presence of extracellular senile plaques containing amyloid β peptides (Aβ) and intracellular neurofibrillary tangles of hyperphosphorylated tau protein [3]. Both structures damage distinct nerve cells, inducing neuronal loss. Hyperphosphorylated tau protein induces changes to the cellular architecture [4] as well as to the localization and organization of subcellular organelles, in turn inducing synaptic interruption and apoptotic neuronal death [5]. In addition, Aβ peptides are one of the principal components of senile plaques and are considered the dominant agents of AD pathogenesis [6]. Aβ peptides are generated from amyloidogenic processing of amyloid precursor protein (APP), which is expressed in many cellular types and tissues, for example endothelial cells, glial cells, and neurons. Indeed, APP participates in intracellular communication and signaling pathways between membrane and nucleus [7]. APP is cleaved

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within the transmembrane domain by γ-secretase to release Aβ peptides, mainly Aβ[1–40] and Aβ[1–42] which constitute the senile plaques. Aβ(1-42) mostly remains in the plaques, because it is hydrophobic and has a mayor aggregation potential. Therefore, this fragment represents the core where Aβ[1–40] is deposited [8]. Moreover, the amino-acid sequence of Aβ can be divided into different functional domains, such as the C-terminus of Aβ(1-42) (which has a fragment of 11 amino-acids) and the (25-35) domain, which is responsible for cytotoxicity and shares with Aβ(1-42) the ability to self-aggregate [9].

The amyloid hypothesis is being proposed to explain AD onset, but it should be underscored that AD depends on the interaction between multiple risk factors, for example age >65 years. Recent epidemiological evidence showed that diabetes is a risk factor for AD onset and development [10–12]. Accordingly, meta-analyses of longitudinal epidemiologic studies show that diabetes increases AD risk by 50–100%; this includes type 2 diabetes, which accounts for 90% of all cases [13,14]. Both disorders share pathophysiological characteristics such as insulin resistance (IR), altered glucose metabolism, oxidative stress and neuroinflammation, amyloid aggregation, neuronal degeneration and cognitive decline, being IR the main binding link between diabetes and AD [15,16]. The insulin-signaling pathway (IR—IRS1/2—PI3K—Akt) is interrupted in IR because regulatory mechanisms are altered. In AD, the insulin receptor substrate-1 (IRS1) is hyperphosphorylated at serine 636/639 and 616 in the hippocampus and cortex of AD patients, which leads to a desensitized insulin signaling pathway because IRS1 changes its conformation and can no longer interact with PI3K, aborting/impeding the activation of Akt by phosphorylation, in turn blocking glucose cellular uptake [17,18]. Therefore, many authors proposed the term "type III diabetes" for AD, precisely because of cerebral IR [19,20]. Moreover, several works indicate that IR, triggered by the inhibitory hyperphosphorylation of IRS1, is positively correlated with Aβ accumulation via an APP processing regulation that favors overproduction, accumulation, and deposition of Aβ, which—in a vicious cycle—further stimulates IR [16,21].

Astrocytes are the foremost cerebral macroglial cells; they are responsible for converting glucose into lactate and transfer it to neurons that use it as fuel [22]. In addition, astrocytes can store glucose as glycogen, which is an important neuronal energy reservoir during period of high activity, for example learning and memory [23]. Therefore, neurons energetically depend on substrates provided by astrocytes and any communication impediment could bear neuronal consequences. Astrogliosis is another hallmark of the AD brain, where reactive astrocytes are surrounding the core of senile plaque and can even penetrate into them [24]. Both oligomers and deposits of Aβ, among others, can trigger astrogliosis in the AD brain [25]. Furthermore, recent studies carried out in human astrocytes have shown that Aβ(1-42) impairs insulin signaling and glycogen storage [26,27].

Recent prospective studies showed that the Mediterranean diet is associated with lower incidence of AD [28] and can improve cognition [29]. Hydroxytyrosol is the preeminent (poly)phenolic component of extra virgin olive oil and is being actively studied for it manifold biological properties [30–32]. In addition to its established activities on the cardiovascular system, HT is being explored in neurodegeneration [33]. As an example, *in vitro* studies have reported that HT is neuroprotective against Aβ-induced neurotoxicity in neuroblastoma N2a cells [34]. Likewise, an *in vivo* study carried out in the AD APP/PS1 mice model has demonstrated that HT improves electroencephalographic activity and mildly benefits cognitive behavior of transgenic mice [35]. However, the effect of HT on IR has been poorly investigated. Pirozzi et al. reported that HT improves insulin sensitivity and glucose tolerance in an animal model of IR and non-alcoholic fatty liver disease [36] and a clinical trial carried out in middle-aged overweight men has suggested that (poly)phenols from olive leaves might improve insulin sensitivity [37].

We hypothesized that HT could exert beneficial effects on IR associated with AD and investigated it mechanisms of action in an astrocytic model of AD.

2. Materials and methods

2.1. Materials

Aβ(25-35) was purchased from Sigma (St. Louis, MO). Aβ(25-35), was dissolved in 9431 μL of sterile PBS and was aged by incubating it at 37°C for 3 days. The solution was aliquoted into 1-mL tubes, sealed with Parafilm M® and stored at −20°C. Hydroxytyrosol was kindly donated by Seprox Biotech (Madrid, Spain) and was dissolved in absolute ethanol to the desired concentrations.

2.2. Cell line and culture conditions

The astrocytic cell line C6 (rat glioma cells) was purchased from American Type Culture Collection in partnership with LGC Standards (Barcelona, Spain). These cells were originally obtained from a rat glial tumor induced by *N*-nitrosomethylurea [38]. Astrocytes were routinely cultured in 100 mm culture dishes in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L D-glucose without phenol red and supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin–streptomycin and glutamine (2 mM). All these reagents were purchased from Thermo Fisher Scientific (Waltham, MA). Cells were grown at 37°C in an atmosphere containing 5% CO_2.

2.3. Cell differentiation and treatment of astrocytes

For all experiments, cells were plated in 60 mm culture dishes (150,000 cells/cm^2) or 96-well plates for 72 h, in DMEM containing 10% (v/v) FBS. Once cells reached 80% confluence, they were differentiated in DMEM, without FBS, but with 0.1 mM cyclic adenosine monophosphate (cAMP) (Sigma, St. Louis, MO). Cultures were maintained for 48 h in this FBS-free differentiation medium, to induce the morphological changes typical of reactive and mature astrocytes [39]. Subsequently,

TABLE 1 *Experimental groups originating from different treatment combinations with hydroxytyrosol (HT) 5 μM for 2 h (pre-treatment) and/or 24 h (post-treatment), and treatment for 24 h with amyloid β peptide (25-35) ($A\beta_{25-35}$) 25 μM*

Treatments	HT pre-treatment for 2 h	$A\beta_{25-35}$ treatment for 24 h	HT post-treatment for 24 h
Experimental groups			
Control 24 h			
2 h HT	X		
$A\beta_{25-35}$		X	
2 h HT + $A\beta_{25-35}$	X	X	
Control 48 h			
24 h HT			X
$A\beta_{25-35}$ + 24 h HT		X	X
2 h HT + $A\beta_{25-35}$ + 24 h HT	X	X	X
Control 24 h and control 48 h groups received no treatment.			

astrocytes were treated with 25 μM of Aβ(25-35) for 24 h, and/or treated with 5 μM of HT for 2 h (pretreatment period) and/or 24 h (post-treatment period) [40], with different combinations giving rise to six experimental groups as shown in Table 1. Astrocytes from control groups received no treatment and were recollected 24 or 48 h after the differentiation period (Table 1). Once cells were differentiated, all subsequent treatments and experiments were carried out in DMEM without FBS. The dose of Aβ(25-35) and treatment times were chosen considering glial fibrillary acidic protein levels as the indicator of reactive astrocytes (data not shown). Likewise, we used HT concentration that should be of physiological relevance [32] and that did not induce cytotoxicity [41]. Three different experiments were performed in duplicate for each assay.

2.4. Cell viability measurements

The MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide, MTT, Sigma) assay is based on the conversion of MTT into formazan crystals by living cells, which determines mitochondrial activity and is widely used as a measure of cell viability. Astrocytes were seeded at a density of 4,000 cells/cm^2 into standard 96-well plates, cultured and treated as described above. After treatments, cells were incubated with 20 μL of MTT reagent (5 mg/mL) for 3 h at 37°C. Afterwards, the media was discarded and the blue formazan crystals were dissolved in DMSO (Applichem GmbH, Germany). The optical density of samples was measured by a microplate reader at 560 nm (Biochrom Asys UVM 340, Cambridge, UK).

2.5. Protein extraction and quantification

Astrocytes were grown and differentiated in 60 mm culture dishes and were exposed to the treatments previously described. The media was discarded and cells were recollected in 200 μL lysis buffer: radioimmunoprecipitation assay buffer (RIPA) buffer (50 mM NaH_2PO_4, 100 mM Na_2HPO_4, 0.1% sodium dodecyl sulfate, 0.5% NaCl, 1% Triton X-100), with 200 mM sodium orthovanadate, 1 mM Pefabloc SC, and 2 mg/mL protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Samples were incubated overnight at −80°C and then centrifuged at 14,000 rpm for 30 min at 4°C to remove debris cellular. The combination of cryogenic conditions with the use of lysis buffer is an excellent method for total extraction, solubilization, and identification of proteins. Clear supernatants were transferred to new tubes to determine total protein concentration by BCA reagent (Pierce, Thermo Fisher Scientific).

2.6. Immunoprecipitation

The associations among targets were studied by immunoprecipitation. After treatments, astrocytes were recollected on ice in 200 μL of lysis buffer pH 7.6 containing 50 mmol/L HEPES, 10 mM EDTA, 50 mmol/L sodium pyrophosphate, 100 mmol/L NaF, 10 mmol/L Na_3VO_4, 1% Triton X-100, 2 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin. Then, 150 μg of protein were immunoprecipitated overnight at 4°C with the corresponding antibody and incubated with protein A-agarose beads (Roche Diagnostics, Indianapolis, IN) for 2 h at 4°C. Immunocomplexes were washed with lysis buffer, extracted for 5 min at 95°C in 4X SDS-PAGE sample buffer (200 mmol/L Tris-HCl, 12% SDS, 4 mmol/L EDTA, 8% 2-mercaptoethanol, 20% glycerol, pH 7.6), and analyzed by Western blotting as described below. All chemicals were purchased from Sigma (St. Louis, MO).

2.7. Western blotting

Western blotting was used to quantify the main components of the insulin-signaling pathway and the associations among selected targets in astrocytes. Total proteins (30 μg) were resolved on a 10% SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were blocked with Tris-buffered saline containing 0.1% Tween 20 (TTBS) containing 5% BSA for 1 h at room temperature. Then, the membranes were incubated with corresponding primary antibodies included phosphorylated (p) anti-p-Thr308-Akt, anti-p-Ser473-Akt, anti-p-mammalian target of rapamycin (mTOR), and their respective total form; anti-Akt and anti-mTOR. All of them were from Cell Signaling Technology (Danvers, MA). Anti-p-Ser636-IRS1, anti-IRS1, anti-regulatory subunit of PI3K (p85), and anti-transporter glucose 2 (GLUT2) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Sigma (St Louis, MO). All membranes were incubated with the primary antibodies (diluted 1:1000) in blocking buffer at 4°C overnight. The membranes were washed three times with TTBS and incubated with the corresponding secondary antibody

TABLE 2 *Cell viability (%) of astrocytes treated with 25 μM Aβ(25-35) and/or 5 μM hydroxytyrosol (HT)*

Experimental groups	*Control 24 h*	*2 h HT*	*Aβ(25-35)*	*2 h HT + Aβ (25-35)*	*Control 48 h*	*24 h HT*	*Aβ (25-35) + 24h HT*	*2h HT+ Aβ(25-35) + 24 h HT*
% Cell viability	100 ± 0.2	104.3 ± 0.6	52.7 ± 4.5*	69.4 ± 2.5	100 ± 0.4	109 ± 1	100.9 ± 0.5#	101 ± 0.3#

The data are percentage of the control ± SEM.
**P < 0.001 versus control 24 h group.*
#P < 0.001 versus Aβ(25-35) group.

conjugated with horseradish peroxidase (Thermo Fisher Scientific, Inc., Waltham, MA) at a dilution of 1:2,000 or 1:5,000 depending on primary antibody, in nonfat milk during 90 min at 25°C. All proteins were detected by chemiluminescence using an ECL system (PerkinElmer Life Sciences, Boston, MA) and quantified by densitometry using Adobe Photoshop's (Adobe systems, Inc., Mountain View, CA). All blots were reblotted with their corresponding total forms or with anti-GAPDH to normalize each sample for gel-loading variability.

2.8. Statistical analysis

We performed one-way ANOVAs, followed by a Bonferroni's test. Values were considered significantly different when the *P* value was < 0.05. Statistical analyses were conducted with the Prism software GraphPad (version 6.00, San Diego, CA). We present data as means ± SD.

3. Results

3.1. Pre- and post-treatment with HT prevents Aβ(25-35)-induced cytotoxicity in astrocytes

We assessed the effects of Aβ(25-35) and HT on astrocyte viability by the MTT assay. After treatment with Aβ(25-35), astrocytes' viability was significantly decreased as compared with controls (Table 2); however, both pre- and post-treatment with HT prevented this effect. The addition of HT alone had no effect on cell viability with respect to controls. Cell supplementation with HT before the addition of Aβ(25-35) did not avoid Aβ(25-35)-induced decreased viability, because cell viability (69.4 ± 2.5%) was significantly lower than of 24-h controls (100 ± 0.2%) (Table 2).

3.2. HT decreases Aβ(25-35)-induced hyperphosphorylation of IRS1

An insulin receptor substrate 1 (IRS1) phosphorylation at serine 636 inhibits the insulin-signaling pathway, leading to insulin resistance (IR) [42,43]. Numerous post-mortem studies have indicated that brains of AD patients exhibit IR, exacerbated by Aβ-induced hyperphosphorylation of IRS1 at serine 636 [17,18]. However, it is not known whether Aβ(25-35) induces this same serine hyperphosphorylation of IRS1 in astrocytes. We found increased pSer636 IRS1 levels in Aβ(25-35)-stimulated astrocytes (Fig. 1). This finding shows, in astrocytes, that Aβ(25-35) is able to mimic the central IR associated with AD. Also, we evaluated the effect of HT on Aβ(25-35)-induced inhibitory hyperphosphorylation of IRS1 in astrocytes by Western blotting assay. Treatment with HT decreased Aβ(25-35)-increased p-Ser636 ISR1 protein levels, to a point below control group levels in the Aβ(25-35)-treated astrocytes supplemented with HT post-treatment group (Fig. 1).

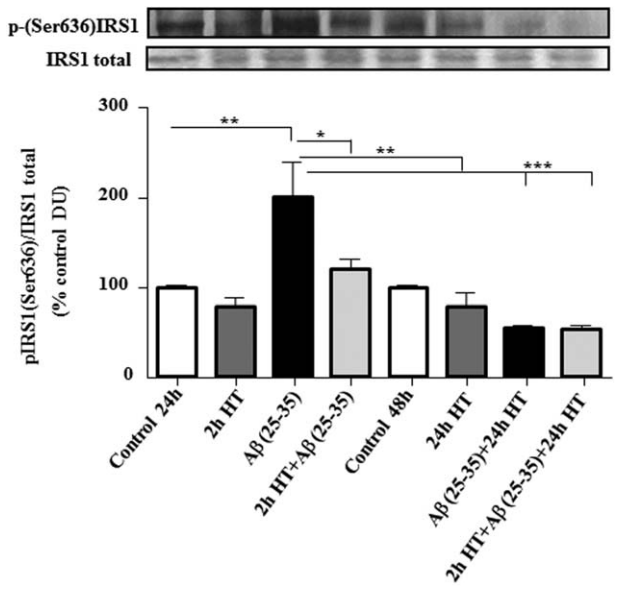

FIG 1 *Densitometry from immunoblots derived from the Western blot analysis of the relative insulin receptor substrate 1 phosphorylated on serine 636 (p(Ser636)-IRS1) protein levels in astrocytes treated with amyloid beta peptide (25-35) (Aβ(25-35)) 25 μM and/or hydroxytyrosol (HT) 5 μM for 2 (pre-treatment) or 24 h (post-treatment), as well as different combinations of both. The data are percentage of the respective control ratio ± SD. DU, densitometry units. **P < 0.01 compared with control 24 h group; *P < 0.05, **P < 0.01, ***P < 0.001 compared with Aβ(25-35) group. Three independent experiments were performed in duplicate.*

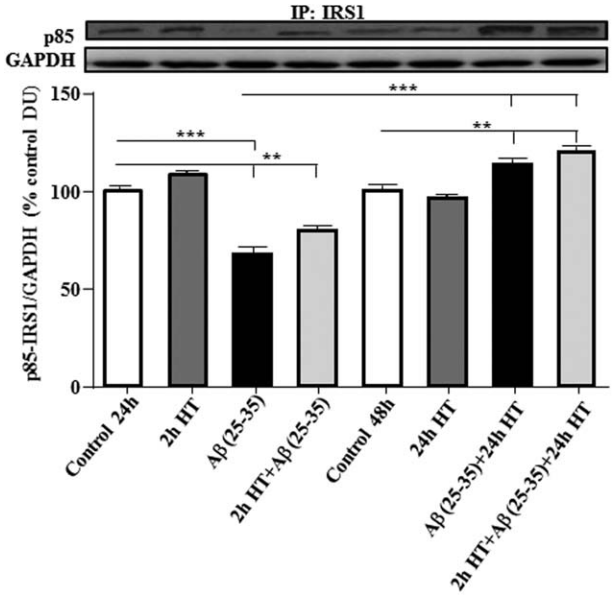

FIG 2

*Densitometric analysis from immunoblots of relative protein levels of the regulatory unit of PI3K (p85) associated with IRS1 in astrocytes treated with amyloid beta peptide (25-35) (Aβ(25-35)) 25 μM and/or hydroxytyrosol (HT) 5 μM for 2 (pre-treatment) or 24 h (post-treatment), as well as different combinations of both. For quantification, p85 associated with IRS1 was measured by immunoprecipitation (IP) of 150 μg of total protein with IRS1 antibody followed by Western blot with anti-p85 antibodies and normalized against GAPDH levels in the same samples. DU, densitometry units. **P < 0.01, ***P < 0.001 compared with control 24 h group; ***P < 0.001 compared with Aβ(25-35) group; **P < 0.01 compared with control 48 h group.*

3.3. HT improves insulin response by increasing the association between p85 and IRS1 in Aβ(25-35)-treated astrocytes

Under normal conditions, after activation of IRS1 by phosphorylation this substrate is bound to the regulatory subunit of PI3K, p85, previous to Akt activation mediated by p110, the subunit catalytic of PI3K. Given that Aβ(25-35) inhibits the insulin signaling pathway-mediated over-activation of Ser636 IRS1 and HT prevented this effect, we evaluated whether HT was able to restore insulin signaling in Aβ(25-35)-treated astrocytes, by immunoprecipitating the association between p85 and IRS1. Immunoblots show that HT post-treatment increased the association between p85 and IRS1 in Aβ(25-35)-treated astrocytes, whereas HT pre-treatment did not restore this association altered by Aβ(25-35) (Fig. 2).

3.4. HT restores proper Akt activation in Aβ(25-35)-treated astrocytes

Akt is a main downstream target of insulin signaling. It is worth noting that Akt phosphorylation at Thr 308 is mediated by glycaemia, which is governed by insulin signaling [44]. Because we observed that HT exerts beneficial effects on Aβ(25-35)-induced upstream of insulin signaling, we also analyzed its effect on Akt activation at Thr308 in astrocytes. Blots indicated that HT increased p-Thr308 Akt protein levels (Fig. 3), which was reduced by Aβ[25–36] in astrocytes, as expected. Treatment with HT alone had no effect on these levels (Fig. 3).

3.5. GLUT2 levels are decreased by Aβ(25-35) in astrocytes, whereas treatment with HT increased them

Metabolically, the main insulin signaling function is to mediate glucose uptake by inducing the glucose transporter isoform 2 (GLUT2) internalization from membrane plasmatic to cytosol [45]. Moreover, GLUT2 is differently expressed in astrocytes [46]. To clarify whether Aβ(25-35) modifies protein expression of GLUT2, we measured protein levels of this transporter in Aβ(25-35)-treated astrocytes and the effects of HT, by Western blotting. Aβ(25-35) reduced GLUT2 levels in astrocytes and treatment with HT prevented this effect (Fig. 4). Notably, a HT pre-treatment appeared to be more effective than post-treatment, as shown by the significantly (as compared with controls) increased GLUT2 levels (Fig. 4).

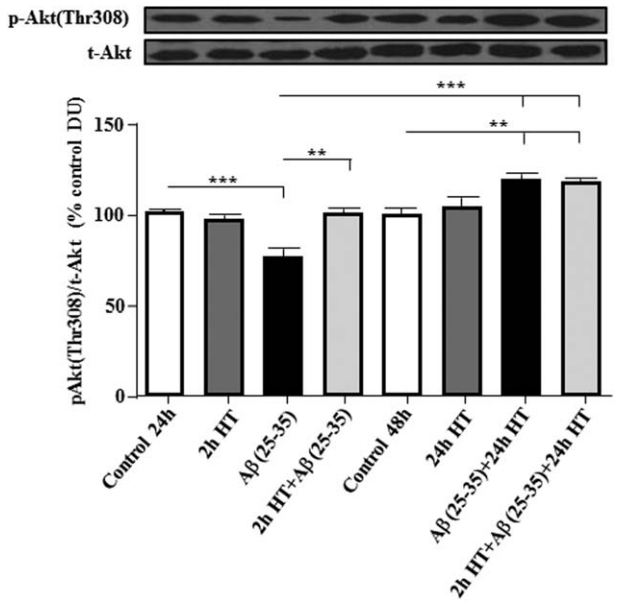

FIG 3

*Western blot and densitometry from immunoblots derived from the relative protein levels of Akt phosphorylated on Thr 308 in astrocytes treated with amyloid beta peptide (25-35) (Aβ(25-35)) 25 μM and/or hydroxytyrosol (HT) 5 μM for 2 (pre-treatment) or 24 h (post-treatment), as well as different combinations of both. Data are percentage of the respective control ratio data ± SD of three independent experiments performed in duplicate. DU, densitometry units. ***P <0.001 compared with control 24 h group; **P < 0.01, ***P < 0.001 compared with Aβ(25-35) group; **P < 0.01 versus control 48 group.*

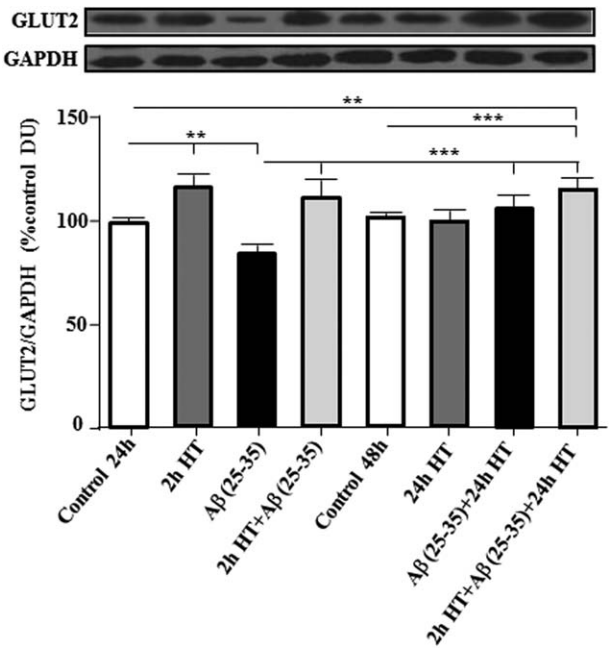

FIG 4

*Western blot and densitometry from immunoblots derived from the protein expression levels of the glucose transporter isoform 2 (GLUT2) in astrocytes treated with amyloid beta peptide (25-35) (Aβ(25-35)) 25 μM and/or hydroxytyrosol (HT) 5 μM for 2 (pretreatment) or 24 h (post-treatment), as well as different combinations of both. Data are percentage of the respective control ratio ± SD of three independent experiments performed in duplicate expressed. DU, densitometry units. **P < 0.01 compared with control 24 h group; and ***P < 0.001 compared with Aβ(25-35) group; ***P < 0.001 compared with control 48 h group.*

3.6. Aβ(25-35)-induced insulin resistance is mediated by the over-activation of mTOR. HT prevented this effect

The mammalian target of rapamycin (mTOR) is a conserved serine/threonine kinase that inhibits insulin signaling by phosphorylating IRS-1 serine residues [47]. Recent studies have shown that mTOR is aberrantly activated in AD brain, which is linked to the insulin signaling inhibition [48]. Because Aβ(25-35) induces IR in astrocytes, we wanted to resolve whether Aβ(25-35) promoted this resistance through an over-activation of mTOR. We thus measured phosphorylated mTOR levels in Aβ(25-35)-treated astrocytes, as well as the effect of HT on them. The observed increased p-mTOR levels (Fig. 5) corroborated the notion that Aβ(25-35) induces IR through of the over-activation of mTOR in astrocytes. Likewise, both pre- and post-treatment with HT prevented this activation in Aβ(25-35)-treated astrocytes (Fig. 5).

4. Discussion

Our main finding is that HT plays both preventive and corrective roles against cytotoxicity and IR induced by Aβ[25] in astrocytes. These data could explain—at the molecular level—epidemiological findings associating EVOO consumption with lower incidence of dementia and AD [49]. To gain insight on the neuroprotective activities of HT, we induced IR with Aβ[25] and we measured cellular viability and proteins pertaining to the insulin signaling pathway targets. The (25-35) fragment of Aβ is widely used in cellular cultures to approximate the astrocytic damage induced by Aβ in the brain of AD patients, because several studies have demonstrated that Aβ is able to pass the cell membrane and exerts cytotoxic effects both on astrocytes and neurons [9,50]. Several authors used our astrocytic "*in vitro*" model of AD (astrocytes treated with Aβ) to assess the effects of different molecules on diverse Aβ-induced damages in astrocytes, for example [51,52]. Moreover, intracerebroventricular injections of Aβ(25-35) cause learning and memory impairments similar to those of AD, in mice [53]. Therefore, we believe we used an appropriate astrocytic *in vitro* model of AD. It is worth clarifying that we used rat instead of human astrocytes because this study aims at providing mechanistic insights into the effect of HT in a murine model of AD (ongoing). The (25-35) domain of Aβ reduced astrocytes viability by ~50%, but treatment (either pre or post)

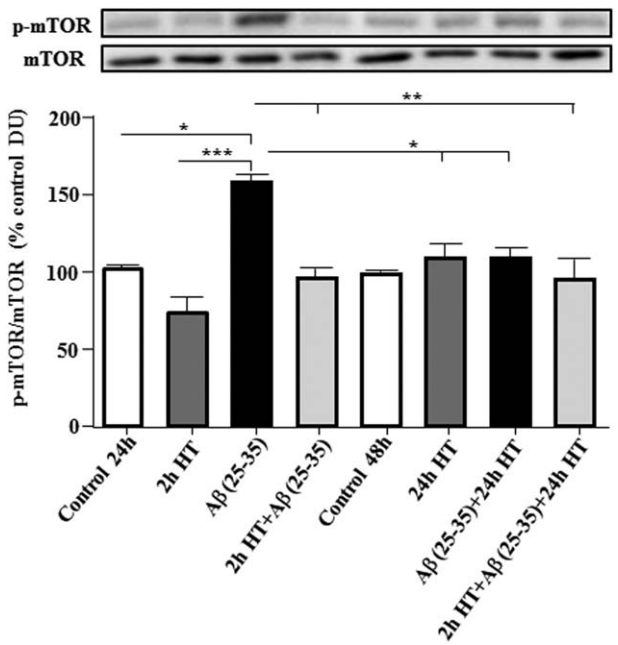

FIG 5

*Immunoblots' densitometry derived from Western blot analysis of the phosphorylated mammalian target of rapamycin (p-mTOR) levels in astrocytes treated with amyloid beta peptide (25-35) (Aβ(25-35)) 25 μM and/or hydroxytyrosol (HT) 5 μM for 2 (pretreatment), or 24 h (post-treatment), as well as different combinations of both. Data are percentage of the respective control ratio ± SD of three independent experiments performed in duplicate. DU, densitometry units. *P < 0.05 compared with control 24 h group; ***P < 0.001 compared with 2 h HT group; *P < 0.05, **P < 0.01 compared with Aβ(25-35) group.*

with HT prevented such cytotoxicity. Neuroprotective effects of HT have been reported before: examples include studies carried out in dopaminergic neurons [54] and in a hypoxia-reoxygenation model of rat brain slices [55]. In addition, a recent study reported that HT improves neuron survival in the brain of db/db mice [56]. However, this is the first time that HT is shown to restore survival in an astrocytic model of AD induced by Aβ(25-35).

As regards IR, Aβ oligomers do impair insulin signaling in human astrocytes [26], but whether the neurotoxic fragment Aβ(25-35) exerts the same alterations has not been clarified yet. In this study, we demonstrated that, indeed, Aβ(25-35) induces IR like the Aβ(1-42) oligomers, because it increases the rate of inhibitory phosphorylation of IRS1, in turn impairing the insulin signaling pathway. In fact, the levels of down-stream targets of insulin signaling such as the associations between p85 and IRS1, pAkt, p-mTOR and also GLUT2 are also reduced by Aβ(25-35), which leads to reduced activity of the whole insulin signaling pathway. Of note, serine hyper-phosphorylation of IRS1 is a marker of IR both in peripheral tissues and in the brain [57,58]. Moreover, several studies unequivocally demonstrated that the IR seen in AD brain is tightly linked to Aβ-induced hyperphosphorylation of IRS1 [17,18]. Nevertheless, treatment with HT significantly reduced Aβ(25-35)-induced hyperphosphorylation of IRS1, suggesting that HT could protect against the Aβ(25-35)-induced IR, thereby augmenting the insulin pathway. To further investigate the effects of HT and to find whether the insulin signaling pathway is activated in astrocytes co-treated with Aβ(25-35) and HT we measured the association between p85 and IRS1 as well as protein levels of down-stream targets of insulin pathway. Indeed, HT treatment prevented the decreased association between p85 and ISR1 in Aβ(25-35)-treated astrocytes, which adds evidence the purported protective activity of HT in IR. The HT-mediated restored association translated in an increased activation of Akt in Aβ(25-35)- and HT-treated astrocytes. Given that the insulin signaling pathway was blocked by Aβ(25-35), Akt activation was also reduced in astrocytes (Figs. 1–3). Along this line, several studies demonstrated that direct exposure to Aβ induced a down-regulation of phospho-Akt at the Thr308 amino acid, in a rat model of induced cognitive decline [59]. In humans, the hippocampus of AD patients shows diminished expression of Akt [27,60].

Several studies also reported increased activation of Akt induced by HT in peripheral tissues. Thus, Pei et al. (2016) have showed that the antiapoptotic and cardioprotective effects of HT on myocardial injury are related to the activation of the Akt/GSK3β pathway [61]. Such HT-induced activation of Akt has been observed in hepatocytes and retinal epithelial cells [62,63]. Hence, various studies support the hypothesis that the protective role of HT is related to activation of Akt. Indeed, Akt is an indispensable component of a great number of cellular signaling pathways involved in cellular survival and prevents cell damage [64]. Therefore, we suggest that the preventive role of HT on Aβ(25-35)-induced cytotoxicity in astrocytes is moderated by an increased HT-induced activation of Akt, which is mediated by the insulin signaling pathway.

The main purpose of insulin signaling in astrocytes is allowing the uptake of glucose through GLUT2 and, indeed, altered glucose transportation has been related to IR in AD patients [65]. In our study, Aβ(25-35) blocked insulin signaling in astrocytes, where we also recorded reduced expression of GLUT2; however, supplementation with HT prevented this reduction. The reduced expression of GLUT2 was specifically induced by Aβ(25-35) and not by glucose in DMEM, because its concentrative was unchanged throughout the whole investigation. In summary, our results suggest that HT could improve glucose metabolism in astrocytes injured by Aβ(25-35). In a broader context, it is worth mentioning that HT is able to lessen some of the consequences of the metabolic syndrome [66–68], which include glucose signaling's derangement.

Our findings show that Aβ(25-35)-induced astrocytic IR is provoked by increased inhibitory phosphorylation of IRS1, which is mediated by the activation of mTOR, among other serine/threonine kinases [47]. Thus, augmented activation of mTOR was found in Aβ(25-35)-treated astrocytes. These high mTOR levels were correlated with increased inhibitory phosphorylation of IRS1 responsible for blocking insulin-signaling pathway in astrocytes. Moreover, over-activated levels of mTOR were reported in Aβ(25-35)-treated hippocampal neurons and in aged mice brain [69]. In support of these findings, other studies have indicated that there is a positive correlation between Aβ levels and mTOR in post-mortem tissues from AD patients [70] and an excessive mTOR activation impaired insulin signaling in hippocampal tissue of rat with diabetes, exacerbating the development of AD [71]. Of note, a recent study by Han et al. [27] did not report increased mTOR levels in HA-1800 cells challenged with $A\beta_{1\text{-}42}$ oligomers and insulin, leaving room for further investigation. In synthesis, reduced glucose utilization is associated with the buildup of Aβ as mediated by over-activated mTOR [48], even though the precise nature of this relation needs to be clarified. Nevertheless, HT prevented the pronounced activation of mTOR in Aβ(25-35) astrocytes, which suggests that HT protects the Aβ(25-35)-blocked insulin signaling pathway.

In conclusion, we demonstrate that HT protects Aβ(25-35)-treated astrocytes by improving insulin sensitivity. These data provide some mechanistic insight on the observed inverse association between Mediterranean diet score (and olive oil consumption) and prevalence of cognitive impairment. Future, ad-hoc human studies will eventually verify whether the use of HT as nutritional supplement could reduce IR associated with AD and, in turn, improve AD symptoms and clinical manifestations.

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Declaration of interest

None

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Chapter 3. *Bioactive Polar Lipids*

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Publication nº 7

Buttermilk and krill oil phospholipids improve hippocampal insulin resistance and synaptic signaling in aged rats.

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Buttermilk and Krill Oil Phospholipids Improve Hippocampal Insulin Resistance and Synaptic Signaling in Aged Rats

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Abstract

Impaired glucose metabolism and mitochondrial decay greatly increase with age, when cognitive decline becomes rampant. No pharmacological or dietary intervention has proven effective, but proper diet and lifestyle do postpone the onset of neurodegeneration and some nutrients are being investigated. We studied insulin signaling, mitochondrial activity and biogenesis, and synaptic signaling in the hippocampus and cortex following dietary supplementation with bioactive phospholipid concentrates of krill oil (KOC), buttermilk fat globule membranes (BMFC), and a combination of both in aged rats. After 3 months of supplementation, although all groups of animals showed clear signs of peripheral insulin resistance, the combination of KOC and BMFC was able to improve peripheral insulin sensitivity. We also explored brain energy balance. Interestingly, the hippocampus of supplemented rats—mainly when supplemented with BMFC or the combination of KOC and BMFC—showed an increase in intracellular adenosine triphosphate (ATP) levels, whereas no difference was observed in the cerebral cortex. Moreover, we found a significant increase of brain-derived neurotrophic factor (BDNF) in the hippocampus of BMFC+KO animals. In summary, dietary supplementation with KOC and/or BMFC improves peripheral and central insulin resistance, suggesting that their administration could delay the onset of these phenomena. Moreover, n-3 fatty acids (FAs) ingested as phospholipids increase BDNF levels favoring an improvement in energy state within neurons and facilitating both mitochondrial and protein synthesis, which are necessary for synaptic plasticity. Thus, dietary supplementation with n-3 FAs could protect local protein synthesis and energy balance within dendrites, favoring neuronal health and delaying cognitive decline associated to age-related disrepair.

Keywords Insulin · Hippocampus · Phospholipids · Buttermilk · Krill oil

Joao Tomé-Carneiro and M. Carmen Crespo contribute equally to this work.

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Abbreviations

AD	Alzheimer's disease
Akt	Protein kinase B
AMPK	AMP-activated protein kinase
α-Syn	Chaperone α-synuclein
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BMFC	Buttermilk fat globule concentrate
CD	Cognitive deficiency
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FAs	Fatty acids
Glut4	Glucose transporter type 4
HOMA-R	Homeostasis model assessment ratio
IRβ	Insulin receptor-beta subunit
IRS	Insulin receptor substrate
KOC	Krill oil concentrate
MFGM	Milk fat globule membrane
mTOR	Mammalian target of rapamycin
PUFA	Polyunsaturated fatty acid
PC	Phosphatidylcholine
PS	Phosphatidylserine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PLE	Pressurized liquid extraction
PI3K	Phosphatidylinositol-3-kinase
PGC-1α	Proliferator-activated receptor γ coactivator 1-α
SM	Sphingomyelin
SIRT1	Sirtuin 1
Stx1A	Syntaxin 1A
Syn1	Synapsin I
Syt1	Synaptotagmin 1
TAG	Triacylglycerides
Vamp2	Synaptobrevin 2

Introduction

Major pathological features of neurodegeneration include impaired glucose metabolism and mitochondrial decay [1, 2]. In particular, insulin governs whole-body energy and peripheral glucose homeostasis but also exerts specific actions in the brain [3, 4], and it is noteworthy that there is increased insulin resistance with age [5, 6]. In addition, dysfunctional operation of the electron transport chain constitutes a key mechanism involved in the age-associated loss of mitochondrial energy metabolism [7].

Insulin signaling in the brain follows the same steps described for peripheral tissues, and its receptor is expressed in neurons and glial cells in different brain regions [8]. Among them, hippocampus and temporal cortex show the highest levels of insulin receptor expression, indicating the important role of insulin in learning and memory [5, 9]. Glucose flux regulates hippocampal memory processing through increased glucose transporter type 4 (Glut4) translocation [10]. Thus, cerebral insulin resistance could severely affect normal cognitive processes. Indeed, risk factors associated with insulin resistance, such as obesity, poor diet, physical inactivity, aging, and genetic predispositions, are correlated with cognitive dysfunction and dementia [11, 12]. Most of these factors are modifiable, pointing to the indispensable roles of prevention.

The Mediterranean diet has been associated with lower incidence of age-related diseases, such as Alzheimer's disease (AD) [13]. For instance, amelioration of insulin signaling was seen after treatment with hydroxytyrosol in an astrocytic model of AD [14], suggesting that the use of extra virgin olive oil might slow down cognitive decline. Moreover, during aging, the central nervous system becomes depleted of the polyunsaturated fatty acid (PUFA) docosahexaenoic acid (DHA) which has been associated with pathologies such as senile dementia and AD [15]. Therefore, DHA supplementation to individuals at risk of cognitive decline could prevent its onset or lessen its consequences.

Currently, there are no dietary recommendations or science-backed health claims concerning cognitive decline [16]. FAO and WHO recommend (for adults) a daily intake of at least 500 mg of eicosapentaenoic acid (EPA) + DHA [17]. However, the current recommendations do not distinguish among fatty fish, functional foods, or supplements/nutraceuticals [18]. Yet, n-3 FAs associated to phospholipids are more bioavailable to different tissues, such as the brain [19]. In this respect, krill oil is an attractive source of n-3 FAs, because a large proportion of them is incorporated into phosphatidylcholine (PC). Beneficial effects of krill oil have been described on blood lipids [20], inflammation [21], and cognitive function in the elderly [22].

Another nutritionally relevant source of polar lipids is the milk fat globule membrane (MFGM). The milk fat globule consists of a core, mainly composed of triacylglycerides (TAG; 98–99%), and different concentrations of other compounds such as diacylglycerides, monoacylglycerides, free fatty acids (FFAs), and cholesterol. This core is surrounded by the MFGM, which contains different phospho- and sphingolipids and that could have potentially positive effects on human health, namely in neurological pathologies [23]. Buttermilk (BM), a by-product obtained from butter manufacturing with a high content of MFGM, is particularly rich, i.e., up to 20% of total fat, in polar lipids [24] and is currently not employed in the supplement arena. This percentage could be further increased by using food-grade solvents to obtain BM lipids and their subfractions [24].

It is noteworthy that BM fractions contain high proportions of phosphatidylserine (PS) and sphingomyelin (SM). The former plays important cellular roles, e.g., in mitochondrial membrane integrity, release of presynaptic neurotransmitters, activity of postsynaptic receptors, and activation of protein kinase C

in memory formation [25]. A decrease of SM in cerebral myelin has been related to the slowing in the speed of the cognitive process associated with aging [26]. For these reasons, there might be an opportunity to incorporate BM fractions in functional foods rich in phospho- and sphingolipids from MFGM, along with short- and long-chain n-3 FAs, e.g., from krill oil to counteract the age-related loss of PUFAs.

Our goal was to study insulin signaling, mitochondrial activity and biogenesis, and synaptic signaling in the hippocampus and cortex following dietary supplementation with bioactive phospholipid concentrates of krill oil (rich in n-3 FAs esterified to PC) and phospho- and sphingolipid concentrates from BM (rich in linolenic acid, PC, phosphatidylethanolamine (PE), phosphatidylinositol (PI), PS, and SM) and a combination of both in aged rats.

Materials and Methods

Materials

Buttermilk and krill oil concentrates (BMFC and KOC, respectively) and the elaboration of daily doses in the form of a jelly lollipop were produced at the Institute of Food Science Research (CIAL, Madrid, Spain). Briefly, BM fat was extracted by pressurized liquid extraction (PLE) using an accelerated solid ASE-200 extractor (Dionex Corp. Sunnyvale, CA). Fifteen grams of powdered BM was mixed with sand (1:1, by weight) and loaded into a stainless steel extraction cell. To obtain the maximum BM fat yield, the extraction procedure was based on the optimized PLE method of Castro-Gomez et al. [27]. The lipid extracts were capped under nitrogen and stored at − 35 °C.

Animals

This research followed the Guide for the Care and Use of Laboratory Animals, published by the US National Research Council (Eight Edition 2010). Animal care was according to the standards proposed by the European Community (86/609/EEC). Animal experiments were approved by the Animal Experimentation Committee of the National University of Distance Education. A total of 41 male Wistar rats (9 months of age) were purchased from Charles River Laboratories (Barcelona, Spain). Rats were randomly distributed in groups of two per cage and maintained in a 12:12 light-dark cycle (8 a.m. to 8 p.m.), with constant temperature and humidity conditions (22 ± 2 °C and 50% RH), during the following 9 months. Food and water were given ad libitum.

Experimental Design and Diets

As 18-month rats are considered old and present a variety of cognitive deficiency (CD) symptoms associated with aging, when animals reached this age, they were randomly assigned to four experimental groups (Table 1). All groups were given a standard EURodent (LabDiet, St. Louis, MO) diet plus a group-specific supplement as frozen strawberry-flavored jellies: (1) control group—refined olive oil ($n = 8$), (2) BMFC group—concentrate of phospho- and sphingolipids of MFGM from buttermilk ($n = 11$), (3) KOC group—concentrate of omega-3 fatty acids (EPA and DHA) and phospholipids from krill oil (KO) ($n = 10$), and (4) BMFC+KOC group—combination, in a single jelly, of the concentrates given to groups 2 and 3 ($n = 12$). The detailed composition of different phospho- and sphingolipids is described in Table 2. Full intake of jellies was visually verified every day.

The nutritional composition of the diet of the four experimental groups is shown in Table 3. Rats were sacrificed by decapitation after 3 months of supplementation, following a 12-h fast. Hippocampus and temporal cortex were quickly extracted, washed in PBS, snap-frozen in liquid nitrogen, and stored at − 80 °C. Blood samples were collected with heparin (100 UI/ml), centrifuged for plasma collection at 1500×g for 15 min, and stored at − 80 °C.

Determination of Circulating Biochemical Parameters

Concentration of plasma glucose was measured with commercial kits (WAKO, Neuss, Germany), and insulin levels were determined by an ELISA kit (Rat Insulin, 96-well plate assay, Millipore, Madrid, Spain), according to the manufacturer's instructions. The insulin resistance index, a.k.a. homeostasis model assessment ratio (HOMA-R), was calculated using the following formula:

$$\mathrm{HOMA} = \text{fasting glucose (mmol/l)} \times \text{fasting insulin } (\mu\mathrm{IU/ml})/22.5.$$

Measurement of ATP Levels in Tissue Samples

To evaluate the energy status of the cells in the hippocampus and temporal cortex, a bioluminescent assay was used to assess intracellular ATP levels (Sigma-Aldrich, St. Louis, MO, USA). The homogenized samples were previously filtered through 0.22-μm filters (Millipore Corp., Bedford, Mass.). The assay was performed according to the manufacturer's protocol, and luminescence was measured at 570 nm using a microplate reader (Biochrom Asys UVM 340, Cambridge, UK).

RNA Isolation and qRT-PCR

Total RNA extractions from both tissues were performed according to the manufacturer's instructions of the miRNeasy Mini kit (Qiagen, Madrid, Spain), including DNA digestion. RNA quantity and purity were analyzed using a NanoDrop

t1.1 **Table 1** Daily supplements assigned to the four experimental groups

Experimental group	Control diet	Daily supplement (jelly)
Control	Ad libitum	70 mg refined olive oil
BMFC	Ad libitum	70 mg of BMFC
KOC	Ad libitum	70 mg of KOC
BMFC+KOC	Ad libitum	70 mg of BMFC + 70 mg of KOC

BMFC, concentrate of phospho- and sphingolipids of milk fat globule membrane from buttermilk; *KOC*, concentrate of omega-3 fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) enriched in phospholipids from krill oil; *BMFC+KOC*, combination of both concentrates

ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., Spain) and RNA integrity using an Agilent's 2100 bioanalyzer. Total RNA was converted into first-strand cDNA using miScript® II Reverse Transcription kit, Qiagen (Izasa, Barcelona, Spain) according to the manufacturer's guidelines.

Electron Transport Chain Array

We used a mitochondrion energy metabolism (SAB Target List) H384 Predesigned 384-well panel (Bio-Rad, Madrid, Spain). Five samples of each experimental group with the highest RNA Integrity Number (RIN) were selected to perform the assay. The predesigned plates were formed by 87 genes, within which 10 were reference genes, 27 genes belonged to complex I, 4 to complex II, 6 to complex III, 15 to complex IV, and 18 to complex V, and 7 were non-exclusively associated with the respiratory chain.

qRT-PCR reactions were performed with miScript SYBR Green PCR kits from Qiagen (Izasa, Madrid, Spain) using a 7900HT Real-Time PCR System (Life Technologies, Alcobendas, Spain). Cycling conditions were a first step of activation at 95 °C for 15 min, 40 cycles of denaturation at 94 °C for 15 s, annealing at 58 °C for 30 s, and a final step for dissociation curve. PPA1 was identified by NormFinder as the most stable reference gene among other candidates.

t2.1 **Table 2** Phospholipid composition of the study supplements

	Control	KOC	BMFC	BMFC+KOC
PE (%)	–	0.55	15.66	5.16
PI (%)	–	–	1.92	0.77
PS (%)	–	–	16.92	6.79
PC (%)	–	99.45	38.74	72.48
SM (%)	–	–	26.76	14.79

BMFC, concentrate of phospho- and sphingolipids of milk fat globule membrane from buttermilk; *KOC*, concentrate of omega-3 fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) enriched in phospholipids from krill oil; *BMFC+KOC*, combination of both concentrates; *PE*, phosphatidylethanolamine; *PI*, phosphatidylinositol; *PS*, phosphatidylserine; *PC*, phosphatidylcholine; *SM*, sphingomyelin. Values are percentage means

Gene Expression of Synaptic Proteins

Expression analyses of genes encoding proteins involved in neural synapses were performed by qRT-PCR in 384-well plates using the same cycling conditions described above. Specific primers for each gene were designed using Primer3 software (Supplementary Table 1). Quantification of relative gene expression was performed using the comparative delta Ct method using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for normalization.

Western Blot Assays

Temporal cortex and hippocampus samples were homogenized with RIPA lysis buffer containing protease and phosphatase inhibitors (Sigma-Aldrich, Madrid, Spain). Homogenates were kept at − 80 °C for 24 h and centrifuged (4 °C, 12,000×*g*, 30 min), and supernatants were collected. Total protein concentrations were determined using BCA protein assay reagent (Thermo Fisher Scientific, Madrid, Spain). Equal (30 μg) amounts of protein were loaded on 10% polyacrylamide gels, submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes (Bio-Rad, Madrid, Spain). Membranes were blocked with 5% non-fat milk powder in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 2 h at room temperature, then incubated overnight at 4 °C with various primary antibodies regarding key components of the insulin signaling pathway and components of the neurotransmissions and neurodegenerations (Supplementary Table 2), and involved in respiratory mitochondrial chain (Abcam ab110413). Following incubation with appropriate secondary antibodies, protein bands were detected by an enhanced chemiluminescence method using the ECL kit (Bio-Rad, Madrid, Spain). Normalization of total protein expression was carried out using GAPDH or corresponding total form in the case of phosphorylated proteins.

Statistical Analyses

Statistical analyses were carried out using GraphPad Prism 7.02 software (GraphPad Software, Inc., La Jolla, CA, USA).

t3.1 **Table 3** Nutritional composition
t3.2 of the diets fed to the four experimental groups

	Lipids (g)	Carbohydrates (g)	Fiber (g)	Proteins (g)	Energy (Kcal)
t3.3 Control	1.82	32.10	2.05	11.01	201.07
t3.4 BMFC	1.82	32.11	2.05	11.01	201.11
t3.5 KOC	1.82	32.10	2.05	11.01	201.07
t3.6 BMFC+KOC	1.89	32.11	2.05	11.01	201.11

BMFC, concentrate of phospho- and sphingolipids of milk fat globule membrane from buttermilk; *KOC*, concentrate of omega-3 fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) enriched in phospholipids from krill oil; *BMFC+KOC*, combination of both concentrates

For gene expression analysis, data were quantified using the ΔΔCt method and fold-change values were reported as $2^{-(\Delta\Delta Ct)}$. Data from independent samples from all experiments were compared by one-way ANOVA, using the Tuckey test to compare all study groups. Values of $p < 0.05$ were considered significant. Results are presented as means ± SEM.

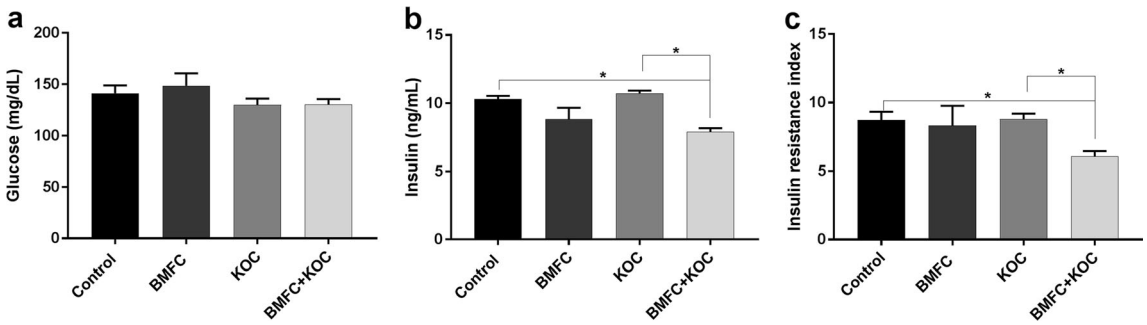
Results

A Combination of BMFC and KOC Improves Peripheral Insulin Sensitivity

Aged rats fed isocaloric diets (Table 3) for 3 months did not show significant changes in body weight (data not shown). As insulin resistance is tightly associated with aging and cognitive dysfunction [28], we wondered whether different dietary supplements affected glycemic profiles. No significant differences in glucose levels in any of the study groups were observed (Fig. 1a). However, glucose levels in all groups (values close to 150 mg/dl) indicated an insulin resistance process, since the normal physiological glucose range of adult (> 4 months) rats is around 75.4 ± 5.5 mg/ml [29]. In addition, serum insulin concentrations were higher than customary for adult rats. Compared to the control group, the combination of BMFC with KOC induced lower insulin levels (Fig. 1b). This observation was further confirmed by the HOMA ratio (Fig. 1c), suggesting that the mixture improves peripheral insulin sensitivity.

Hippocampal and Cortical Insulin Signaling Is Increased in High Phospholipid Concentrate Diets

Dysregulation of brain insulin signaling is associated with aging and neurodegenerative disorders [3, 4, 11]. To determine whether the different supplements influence central insulin signaling, we evaluated key insulin pathway molecules in the hippocampus and cerebral cortex. Protein analysis from hippocampal samples showed a significant increase in the insulin receptor-beta subunit (IRβ) expression levels both in KOC and BMFC+KOC groups (Fig. 2a), whereas the activated insulin receptor substrate (IRS) 1 was augmented in those groups supplemented with BMFC. This activation mediated by the insulin receptor was not observed in the IRS2 protein. Since cytoplasmic insulin signaling is mediated through phosphatidylinositol-3-kinase (PI3K), we also explored the expression levels of the regulatory subunit p85 from this kinase. As seen for IRS1, BMFC supplementation increased PI3K expression levels. As the last step of the insulin pathway, i.e., protein kinase B (Akt) activation, was detected in hippocampal samples of the three experimental groups, it is conceivable that a high-phospholipid diet improves hippocampal



Q5 **Fig. 1** Determination of insulin, glucose, and insulin resistance index in rat serum. BMFC concentrate of phospho- and sphingolipids of milk fat globule membrane from buttermilk; KOC concentrate of omega-3 fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) enriched in phospholipids from krill oil; BMFC+KOC combination of both concentrates. **a** Glucose levels expressed in mg/ml. **b** Insulin levels expressed in mg/ml. **c** homeostasis model assessment ratio (HOMA-R), which is an index of insulin resistance. Values are means ± SEM. *Statistically significant difference regarding all study groups at $p < 0.05$

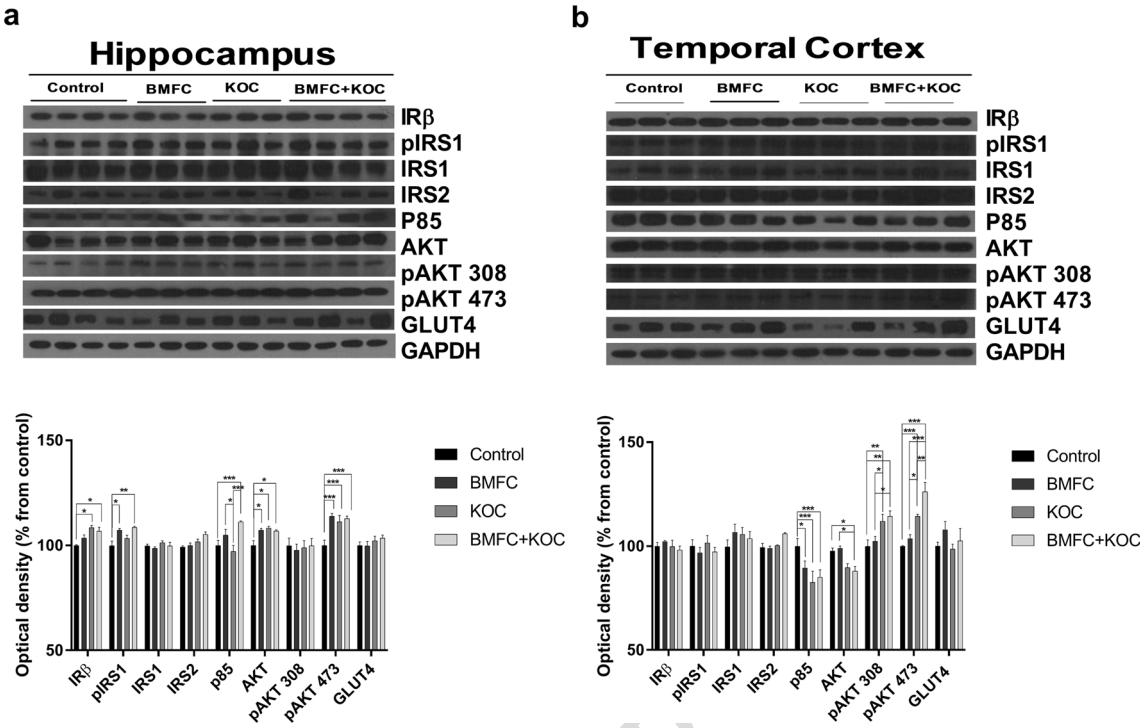


Fig. 2 Western blot of proteins involved in the insulin-signaling pathway. **a** Hippocampus and **b** temporal cortex. IRβ insulin receptor-beta subunit; pIRS1 phosphorylated insulin receptor substrate 1; IRS1 insulin receptor substrate 1; IRS2 insulin receptor substrate 2; p85 subunit of phosphatidylinositol 3-kinase (PI3K); Akt serine/threonine protein kinase; pAkt 308 phosphorylated Akt threonine 308; pAkt 473 phosphorylated Akt serine 473; GLUT4 glucose transporter 4; BMFC concentrate of phospho- and sphingolipids of milk fat globule membrane from buttermilk; KOC concentrate of omega-3 fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) enriched in phospholipids from krill oil; BMFC+KOC combination of both concentrates. Values are means ± SEM. Statistically significant difference regarding all study groups at $*p < 0.05$–0.005; $**p < 0.005$–0.0005; $***p < 0.0005$

insulin signaling. Moreover, cerebral cortex Akt is also activated (Fig. 2b), suggesting that this effect is not restricted to one brain region.

Activation of insulin intracellular signaling stimulates the translocation of the Glut4 transporter facilitating glucose uptake [11]. Although we did not find significant differences in Glut4 levels among any of the supplemented groups (Fig. 2a, b), the implication of other glucose transporters cannot be ruled out.

BMFC+KOC Ameliorate Cellular Energy States and Increase Mitochondrial Biogenesis in Hippocampus

The energetic state of the brain is influenced by mitochondrial biogenesis, which can be dramatically damaged during aging. Thus, we first evaluated the intracellular levels of adenosine triphosphate (ATP) in hippocampal samples. We found that all experimental groups showed an increase in ATP levels, although statistical significance was only reached in BMFC-supplemented animals (Fig. 3a). Interestingly, this improvement was not detected in cerebral cortex samples (Fig. 3b), suggesting that supplementation with bioactive phospholipids differentially affects brain areas at this level. Consequently, we wanted to figure out whether this potentially increased hippocampal energetic state could be related to augmented mitochondrial biogenesis. To achieve this, we analyzed the expression of proteins from the five electron transport chain complexes (Fig. 3c). Animals fed with both BMFC and KOC supplement showed increased protein expression in all complexes studied, suggesting that amelioration of cell energy state could be mediated by an increase in hippocampal mitochondrial biogenesis.

We also evaluated the expression of 70 genes corresponding to the five mitochondrial complexes and seven associated with the respiratory chain (not included in any complex) in the hippocampus. An increase in the expression of key genes involved in the complexes I, IV, and V (Fig. 3d) was detected by qRT-PCR, confirming the observed changes in protein levels.

Mitochondrial Biogenesis in BMFC+KOC-Supplemented Animals Is Mediated Through BDNF

To determine whether the increased levels in mitochondrial complex proteins were mediated by the regulator of mitochondrial biogenesis, i.e., the peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α), we explored the levels of

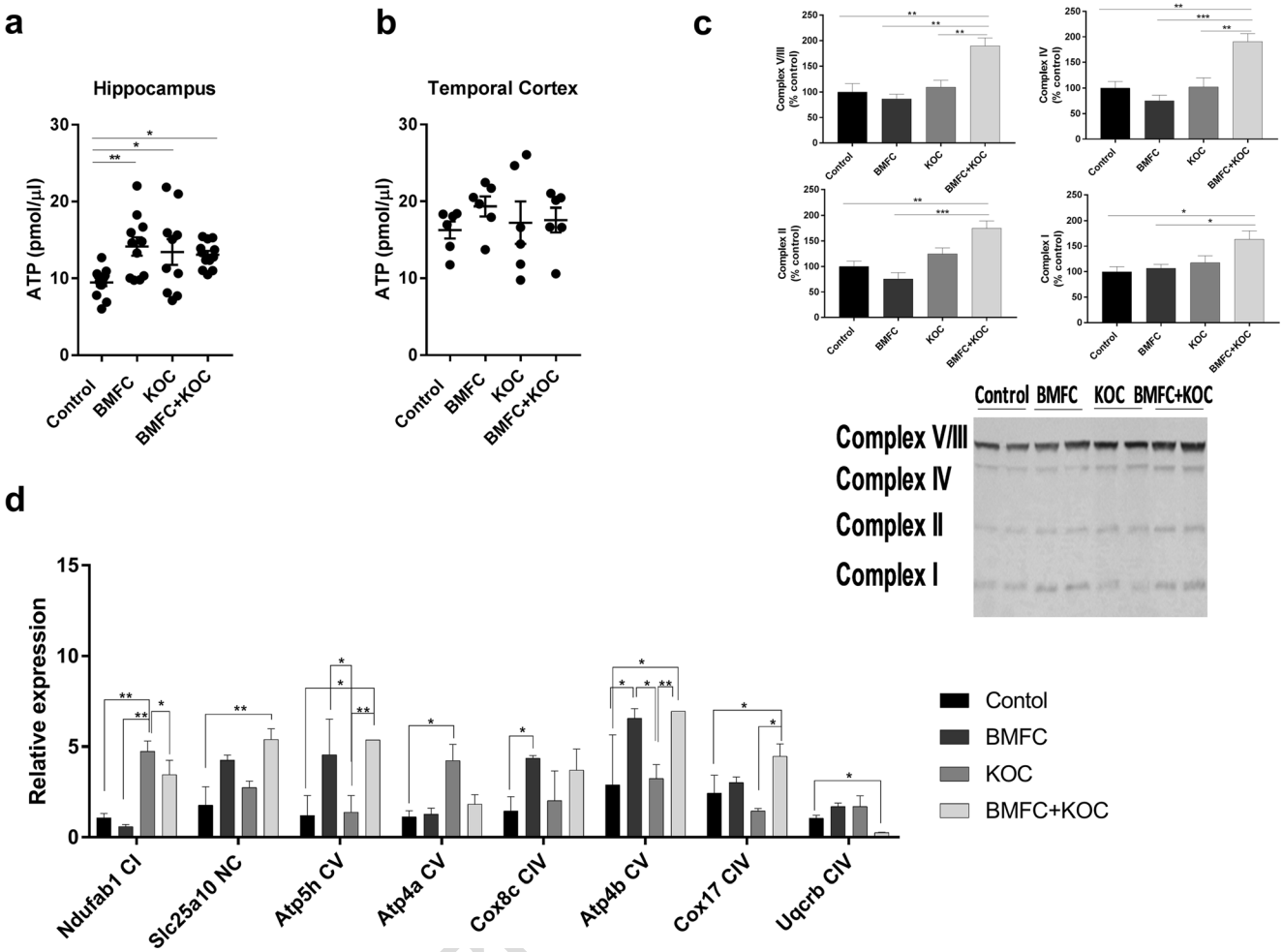


Fig. 3 Evaluation of cellular energy status and mitochondrial biogenesis. **a**, **b** ATP levels expressed in pmol/µl. **c** Western blot analysis of proteins involved in the hippocampal respiratory mitochondrial chain. **d** Hippocampal gene expression regarding the mitochondrial respiratory chain pathway. ATP adenosine triphosphate; Ndufab1 NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex 1; Slc25a10 solute carrier family 25 (mitochondrial carrier, dicarboxylate transporter) member 10; Atp5h ATP synthase, H+ transporting, mitochondrial F0 complex, subunit D; Atp4a ATPase H+/K+ transporting alpha subunit; Cox8c cytochrome c oxidase subunit 8C; Atp4b ATPase H+/K+ transporting beta subunit; Cox17 cytochrome c oxidase copper chaperone; Uqcrb ubiquinol-cytochrome c reductase binding protein; CI complex I; NC does not belong to any complex; CIV complex IV; CV complex V; BMFC concentrate of phospho- and sphingolipids of milk fat globule membrane from buttermilk; KOC concentrate of omega-3 fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) enriched in phospholipids from krill oil; BMFC+KOC combination of both concentrates. Values are means ± SEM. Statistically significant difference regarding all study groups at $*p < 0.05$–0.005; $**p < 0.005$–0.0005; $***p < 0.0005$

this protein in hippocampal and cortical samples (Fig. 4a, b). As seen in Fig. 4a, b, PGC1-α was increased in BMFC+KOC-supplemented rats in both brain areas. As this coactivator is regulated by the master metabolic regulator 5' AMP-activated protein kinase (AMPK), we further explored the levels of this kinase along with the NAD^+-dependent type III deacetylase sirtuin 1 (SIRT1) [30]. Remarkably, although phosphorylated levels of AMPK were only increased in the KOC group both in hippocampus and cerebral cortex, SIRT1 levels were decreased in all experimental groups in the hippocampus and a trend (not significant) was observed in the cortex. As it has been described that PGC-1α activation requires both AMPK and SIRT1 [30], results suggest that AMPK-SIRT1-mediated PGC1-α regulation is not taking place in this model.

Brain-derived neurotrophic factor (BDNF) has been implicated in neural ATP enhancement and in PGC-1α activation [31]. Thus, we next analyzed BDNF levels in hippocampal samples (Fig. 4a). Only BMFC+KOC-supplemented animals showed an increase in BDNF levels, in accordance with the results for PGC-1α. However, although cortical BDNF was increased in all supplemented groups, statistical significance was not reached, suggesting that the amelioration of the energy state observed in the hippocampus of BMFC+KOC-supplemented rats could be mediated by BDNF. Some authors propose that BDNF acts through the mammalian target of rapamycin (mTOR), favoring local protein synthesis in dendrites [32]. Increased levels of mTOR activity were detected in rats supplemented with BMFC+KOC, suggesting a

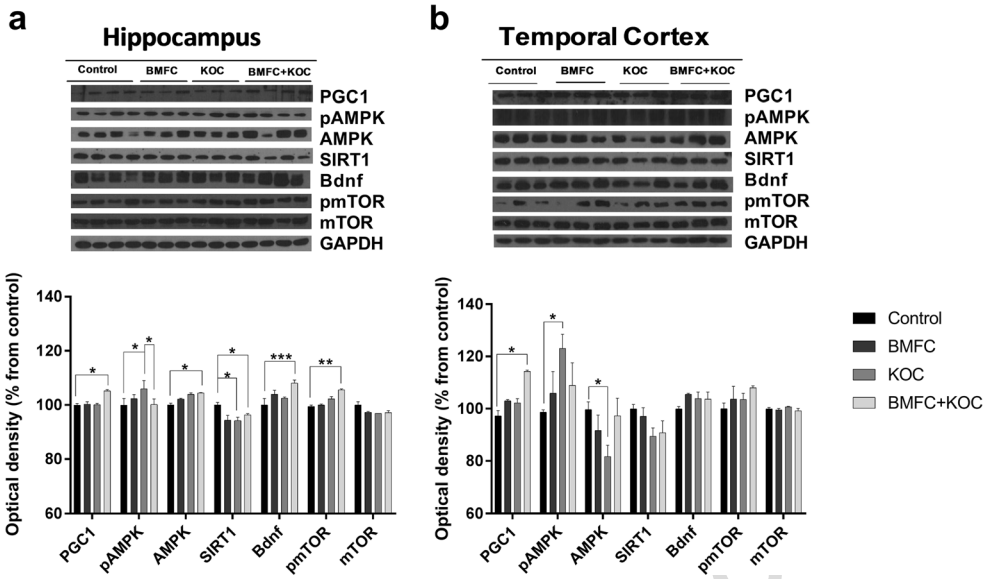


Fig. 4 Western blot of proteins involved in mitochondrial biogenesis. **a** Hippocampus and **b** temporal cortex. PGC1 peroxisome proliferative activated receptor, gamma, coactivator 1 alpha; pAMPK phosphorylated AMPK; AMPK AMP-activated protein kinase; SIRT1 sirtuin 1; BDNF brain-derived neurotrophic factor; pmTOR phosphorylated mechanistic target of rapamycin kinase; mTOR mechanistic target of rapamycin kinase; BMFC concentrate of phospho- and sphingolipids of milk fat globule membrane from buttermilk; KOC concentrate of omega-3 fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) enriched in phospholipids from krill oil; BMFC+KOC combination of both concentrates. Values are means ± SEM. Statistically significant difference regarding all study groups at $*p < 0.05$–0.005; $**p < 0.005$–0.0005; $***p < 0.0005$

possible BDNF-associated role of mTOR in the improvement of hippocampal energy state.

Synaptic Proteins Are Differentially Regulated in Distinct Brain Areas

Considering that mitochondrial biogenesis is relevant for BDNF to stimulate the formation of new synapses and to maintain the existing ones [31], we wondered whether the observed changes in energy status after supplementation could be linked to synaptic signaling. Hence, we first analyzed the expression levels of genes encoding pre- and postsynaptic proteins in hippocampal samples (Fig. 5a). Interestingly, syntaxin 1A (*Stx1A*) gene levels were increased in BMFC+KOC-supplemented animals, whereas synapsin I (*Syn1*) and synaptotagmin 1 (*Syt1*) did not change. We further examined postsynaptic structural proteins PDS95, PDS93, and SAP-102 levels (*Dlg4*, *Dlg2*, and *Dlg3*, respectively), and, surprisingly, while *Dlg4* levels were increased in KOC rats, the expression of all three genes was decreased in the BMFC+KOC group. In order to confirm these results, we applied Western blot techniques to the protein Stx1A and two other presynaptic proteins associated with synaptic vesicles, synaptobrevin 2 (Vamp2) and the chaperone α-synuclein (α-Syn) (Fig. 5b). Even though *Stx1A* mRNA levels were increased in the BMFC+KOC-supplemented animals, protein levels were not changed in any of the experimental groups. On the other hand, Vamp-2 levels were increased in all supplemented animals.

In the case of cerebral cortex, some of the pre- and postsynaptic proteins studied increased gene expression levels (Fig. 5c). *Stx1A* levels were significantly increased in BMFC+KOC-supplemented rats, whereas *Syt1* mRNA was upregulated in all studied groups compared to that in control animals. At protein level (Fig. 5d), Stx1a and α-Syn were increased in the KOC group and α-Syn was augmented only in the BMFC group. These results highlight different synaptic regulations in distinct brain areas.

Discussion

There is growing evidence that age-associated cognitive decline can be partially prevented by an appropriate lifestyle that includes proper diet and regular exercise [33, 34]. One example is that of the Mediterranean diet, which has been correlated with lower incidence of age-related disorders, such as AD and cardiovascular disease [35, 36]. Dietary fats such as those of fish or olive oil could be responsible for such effects by ameliorating, for instance, insulin signaling in brain [14]. Dietary supplementation with PUFAs, such as DHA or the more bioavailable n-3 FAs associated to phospholipids, present in krill oil, could represent a good pharma-nutritional tool to partially prevent the noxious consequences of age [19, 22]. Indeed, milk phospholipid intake seems to have beneficial effects on stress-induced memory impairment in adults [37]. Although MFGM supplementation in infant and children appears to have some neurodevelopmental

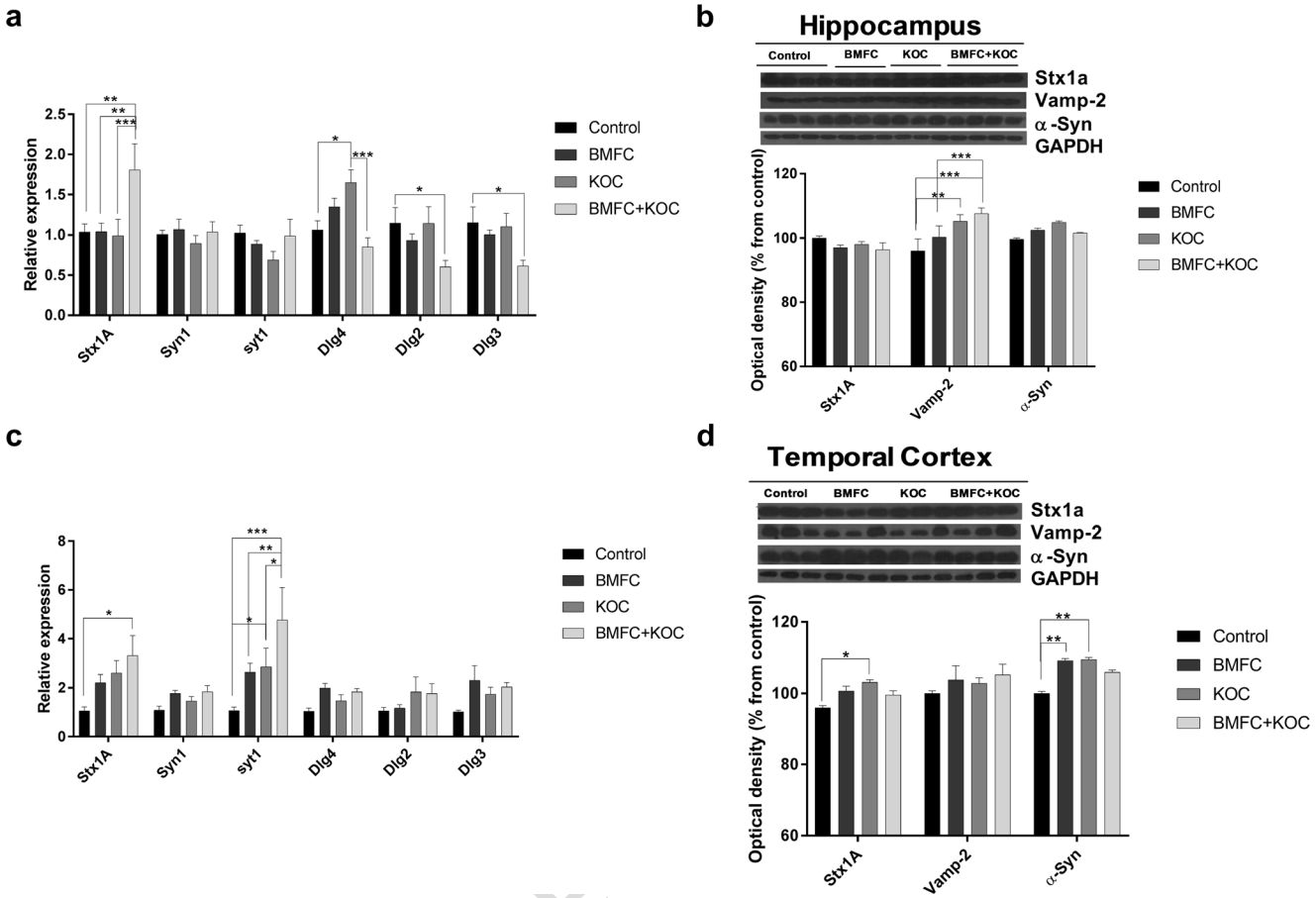


Fig. 5 Synaptic protein expression in both tissues. **a** Hippocampal expression analysis of genes encoding for proteins involved in the nerve synapse. **b** Western blots of hippocampal proteins involved in neurotransmission signaling. **c** Temporal cortex expression analysis of genes encoding for proteins involved in the nerve synapse. **d** Western blot of temporal cortex proteins involved in neurotransmission signaling. STX1A syntaxin 1A; SYN synapsin I; SYT1 synaptotagmin 1; Dlg4 discs large MAGUK scaffold protein 4 (PSD95); Dlg2 discs large MAGUK scaffold protein 2 (PSD93); Dlg3 discs large MAGUK scaffold protein 3 (SAP-102); Vamp-2 vesicle-associated membrane protein 2; α-Syn synuclein alpha; BMFC concentrate of phospho- and sphingolipids of milk fat globule membrane from buttermilk; KOC concentrate of omega-3 fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) enriched in phospholipids from krill oil; BMFC+KOC combination of both concentrates. Values are means ± SEM. Statistically significant difference regarding all study groups at $*p < 0.05–0.005$; $**p < 0.005–0.0005$; $***p < 0.0005$

benefits [38], their effects on the elderly are insufficiently investigated [39, 40].

As insulin resistance increases with age and its associated diseases [5], we aimed at studying brain insulin signaling in response to dietary supplementation with bioactive phospholipid concentrates of krill oil and buttermilk in aged rats. After 3 months of supplementation, although all groups of animals showed clear signs of peripheral insulin resistance, the combination of KOC and BMFC was able to improve peripheral insulin sensitivity. In agreement, both krill oil and MFGM have been previously shown to reduce insulin levels in type 2 diabetes and obese individuals, respectively [41, 42], suggesting that dietary fatty acids/phospholipids could help in this respect. In order to know whether this amelioration in peripheral insulin resistance could also be detected in the central nervous system, we further explored insulin signaling in the hippocampus and cerebral cortex, both implicated in cognitive processes. Interestingly, insulin signaling was improved in both structures, since the cascade of events was active when animals were supplemented with BMFC, KOC, or both. These results highlight the potential of dietary fats to compensate the disturbance in the insulin pathway observed with aging.

Considering that the energetic state level in brain is influenced by its mitochondrial content, we further explored brain energy balance. Interestingly, the hippocampus of supplemented rats—mainly with BMFC or the combination of KOC and BMFC—showed an increase in ATP levels, whereas no difference was observed in the cerebral cortex, suggesting that dietary fats induce different energy states in distinct brain areas, which is coherent with the observation that n-3 fatty acids incorporate into the various brain structures in a non-random, selected fashion [43]. The increased energy status of the hippocampus might be due to an increase in mitochondrial biogenesis, because both mitochondrial complex

proteins and PGC-1α, the key regulator of mitochondrial biogenesis, were augmented in BMFC+KOC-supplemented animals. Remarkably, although we did not record higher ATP levels in the cerebral cortex of supplemented animals, BMFC+KOC rats presented increased levels of PGC-1α, suggesting that the combination of the two types of fat favors the increase of this transcription factor, in turn indicating an increase in mitochondrial biogenesis signaling in this brain area. Since all treated animals presented increased levels of the neurotrophin BDNF, upregulation of PGC-1α appears to be mediated through it rather than AMPK-SIRT1. However, we only found a significant increase of BDNF in the hippocampus of BMFC+KOC animals. It is noteworthy that cerebral BDNF levels are quite low [31] and perhaps even a slight rise in BDNF levels could promote the action of PGC-1α in the cerebral cortex.

BDNF stimulates mitochondrial biogenesis to form new synapses and to maintain the existing ones [31]. In fact, dendritic protein synthesis is indispensable to sustain synaptic plasticity, where BDNF is the main modulator [32]. BDNF facilitates synaptogenesis by inducing the mTOR pathway, which mediates signals for local protein synthesis [32]. According to our results, combined supplementation with both KOC and BMFC favored mTOR activation in the hippocampus. Thus, animals fed with both fats exhibit all the necessary steps for synapse formation [32, 44].

Although our results point to an improvement in synaptogenesis in hippocampal samples of BMFC+KOC-supplemented animals, when we explored synaptic genes and proteins, we did not find significant increases in this brain area. Surprisingly, cerebral cortices presented an induction of synaptic protein synthesis in all supplemented groups, although the highest levels were detected in the BMFC+KOC-supplemented group. As mentioned above, although BDNF and mTOR levels increased, statistical significance was not seen regarding controls, but it may be possible that such increase is enough to improve synaptic protein synthesis at the dendrite level.

In summary, dietary supplementation with KOC and/or BMFC improves peripheral and central insulin resistance and suggests that it could help delay their onset. Moreover, as described before [45], n-3 fatty acids ingested as phospholipids increase BDNF brain levels, favoring an improvement in energy state within neurons and facilitating both mitochondrial and protein synthesis, which are necessary for synaptic plasticity. Thus, dietary supplementation with n-3 FAs could protect local protein synthesis and energy balance within dendrites, favoring neuronal health and delaying cognitive decline associated to age-related disrepair.

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Author Contributions JF, CV, and FV designed the study. JTC, MCC, EBR, IPP, SB AGS, PCG, and AV performed experiments. MCC, JTC, EBR, CTZ, AD, and FV wrote the manuscript. All authors approved the submission of the final version of the manuscript.

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Compliance with Ethical Standard

Animal experiments were approved by the Animal Experimentation Committee of the National University of Distance Education. Q6

Conflict of Interest The authors declare that they have no conflict of interest.

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Electronic supplementary material

Supplementary Table 1. Sequence of synapto-proteins primers analyzed.

Gene		Primers sequences
Presynaptic	Stx1A	F: 5′- GCCCTGTTTGATTACGACAA-3′
		R: 5′- ACTCGGTTCTGAGCTATGAG
	Syn1	F: 5′- TGTCCCGAAAGTTTGTG-3′
		R: 5′- GCGTTCTCGGTAGTCT
	Syt1	F: 5′- CTTCTCCAAGCACGACATCA-3′
		R: 5′- CCACCCACATCCATCTTCTT -3′
Postsynaptic	Dlg4	F: 5′- TAGGGCCCTGTTTGATTACG-3′
		R: 5′- TGGCCTTTAACCTTGACCAC -3′
	Dlg3	F: 5′- GAGTTCCCGCATAAGTTTGG-3
		R: 5′- CGGACACGTCTAAGATGCAG -3′
	Dlg2	F: 5′- GTCGGAGGTTTCCCACAGTA-3′
		R: 5′- CTGTGCAGCTCCACCATCTA -3′

Stx1A: syntaxin 1A; Syn: synapsin I; Syt1: synaptotagmin 1; Dlg4: discs large MAGUK scaffold protein 4; Dlg2: discs large MAGUK scaffold protein 2; Dlg3: discs large MAGUK scaffold protein 3; F: Forward; R: Reverse.

Supplementary Table 2. List of the primary antibodies 664 used to analyze insulin signaling pathways, neurotransmission, and neurodegeneration.

Antibodies	*Molecular Weight (kDa)*	*Host*	*Company*
p-thr308-AKT	60	Rabbit	Cell Signaling *
p-thr473-AKT	60	Rabbit	Cell Signaling *
AKT	60	Rabbit	Cell Signaling *
p-mTOR	289	Rabbit	Cell Signaling *
mTOR	289	Rabbit	Cell Signaling *
p-IRS1	90	Rabbit	Santa Cruz Biotechnology **
IRS1	90	Rabbit	Santa Cruz Biotechnology **
IRS2	185	Mouse	Millipore *
IRβ	75-100	Mouse	Santa Cruz Biotechnology **
p85 PI3K	75-85	Mouse	Santa Cruz Biotechnology **
p-AMPK α1/2	60	Rabbit	Santa Cruz Biotechnology **
AMPK α1/2	63	Mouse	Santa Cruz Biotechnology **
SIRT1	120	Rabbit	Santa Cruz Biotechnology **
PGC1	90	Rabbit	Santa Cruz Biotechnology **
GLUT2	55-60	Rabbit	AD Internacional ***
GLUT4	50-63	Mouse	Santa Cruz Biotechnology **
VAMP-2	18	Mouse	Santa Cruz Biotechnology **
Syntaxin 1	35	Mouse	Santa Cruz Biotechnology **
p-TAU	50-80	Mouse	Cell Signaling *
TAU-5	46-80	Mouse	Santa Cruz Biotechnology **
α-synuclein	19	Mouse	Santa Cruz Biotechnology **
BDNF	14	Mouse	Novusbio ****
UBB	9	Mouse	Millipore *

*(Madrid, Spain); **(Heidelberg, Germany); ***(San Antonio, USA); ****(Abingdon, 667 United Kingdom); ***** (Cambridge, United Kingdom).

Publication nº 8

Modulation of miRNA expression in aged rat hippocampus by buttermilk and krill oil.

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Modulation of miRNA expression in aged rat hippocampus by buttermilk and krill oil

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Running title: BMFC + krill oil influence hippocampus miRNA-gene interaction

Keywords: miRNA, hippocampus, phospholipids, buttermilk, krill oil

Abstract

The increasing incidence of age-induced cognitive decline justifies the search for complementary ways of prevention or delay. We studied the effects of concentrates of phospholipids, sphingolipids, and/or 3-n fatty acids on the expression of genes or miRNAs related to synaptic activity and/or neurodegeneration, in the hippocampus of aged Wistar rats following a 3-month supplementation. The combination of two phospholipidic concentrates of krill oil (KOC) and buttermilk (BMFC) origin modulated the hippocampal expression of 119 miRNAs (11 were common to both BMFC and BMFC+KOC groups). miR-191a-5p and miR-29a-3p changed significantly only in the BMFC group, whereas miR-195-3p and miR-148a-5p did so only in the combined-supplemented group. Thirty-eight, 58, and 72 differentially expressed genes (DEG) were found in the groups supplemented with KOC, BMFC and BMFC + KOC, respectively. Interaction analysis unveiled networks of selected miRNAs with their potential target genes. DEG found in the KOC and BMFC groups were mainly involved in neuroactive processes, whereas they were associated with lysosomes and mRNA surveillance pathways in the BMFC + KOC group. We also report a significant reduction in hippocampal ceramide levels with BMFC + KOC. Our results encourage additional in-depth investigations regarding the potential beneficial effects of these compounds.

Introduction

According to the most recent Global Burden of Disease Study 2015 [1], the burden of neurological disorders has increased substantially over the past 25 years because of expanding population numbers and ageing. Even though the prevalence of communicable neurological disorders decreases, the number of patients who will need care by neurologists will continue to grow in coming decades. Indeed, mostly because of the lack of suitable animal models, neurodegeneration – including cognitive decline - is currently difficult to treat and poses an economical and sociological burden on the national health care systems. Disease-unrelated memory decline increases with age in rodents and humans and appears to be associated with subtle changes in the connectional and functional integrity of key hippocampal circuits [2-4]. Emphasizing the importance of this brain region, several gene transcriptional changes influence a wide range of learning and memory-related processes in the hippocampus of aged rats with memory impairment [5]. Moreover, these processes - including neurogenesis, synaptic plasticity and neuronal connectivity - are affected by nutrition [6]. In this respect, the Mediterranean diet has been suggested as being neuroprotective [7]. Its effects are mediated by changes in the expression of multiple genes and associated regulatory networks; nutrition-gene interactions play important roles in optimal and sub-optimal cognitive function. Epigenetics is also emerging as an important mechanism through which nutrition can directly influence the genome [8]. Numerous epigenetic processes are involved in complex mechanisms of gene regulation, as is the case of synaptic plasticity, learning, and memory. Among other players, these processes include changes in non-protein-coding RNAs (ncRNAs) [8,9]. MicroRNAs (miRNAs), small ncRNAs, which post transcriptionally repress the expression of target genes, have the potential to be used as biomarkers of neurodegenerative disease [10].

Age-related loss of cerebral n-3 fatty acids (FAs) and polar lipids (PL) together with low intakes of these compounds (which are essential for the activity, functioning, and maintenance of the nervous system) is related to a greater risk of neurodegenerative diseases [11]. The ISSFAL, the World Health Organization (WHO) and the Food and Agricultural Organization (FAO) recommend (for adults) a daily intake of at least 500 mg of eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) [12]. In this respect, krill oil is an attractive source of n-3 FAs incorporated into phosphatidylcholine (PC). In addition, buttermilk (BM), a by-product obtained from butter manufacturing with a high content of milk fat globule membrane (MFGM), is rich in polar lipids, particularly phosphatidylserine (PS) and sphingomyelin (SM), with potential positive effects in neurological pathologies [13] and is currently not employed in the supplement arena. The nutritional recommendations of physiological doses indicated for an adult of phosphatidylserine are 300-500 mg/d.

In this study, we evaluated whether buttermilk and krill oil as sources of essential fatty acids modulate the expression of miRNAs and their target genes in the hippocampus, due to its importance in neurodegenerative processes, of aged rats.

Results

Supplementation with polar lipids does not affect body weight

To determine whether the consumption of different supplements (**Table 1**) influences body weight, rats were weighted every week during the three months of supplementation. We did not find any significant difference in body weight throughout the study (**Figure 1**). The different diets contained similar caloric composition (**Table 2**) but differ on the types of fatty acids (**Table 3**).

Blood tests

We studied selected biological markers to see if supplements had effects on the lipid profile, namely on cholesterol, triglyceride, non-esterified fatty acid, and phospholipids concentrations. There were no significant differences between the groups in the lipoprotein profile as analyzed by FPLC (**Figure 2A**); significant differences were found in phospholipid levels, which were decreased in the BMFC and BMFC + KOC groups as compared with controls (**Figure 2B**). These decreases could be due to a compensatory physiological mechanism in response to the extra supply of phospholipids. We did not find significant changes between groups in the other parameters analyzed.

Ceramide levels are reduced by BMFC + krill oil supplementation

Possible effects of dietetic supplementations on the composition of key lipids were assessed in the hippocampus. No major differences in lipids classes were observed (results not shown), except for ceramides, whose level was significantly reduced in the BMFC + KOC group (**Figure 3**).

Supplementation with phospholipid concentrates modulates the expression of selected miRNAs

As epigenetic regulation of gene expression is highly flexible in the brain [14] and miRNAs – considered epigenetic markers – are involved in brain development and cognition [10], we assessed the expression of miRNAs in the hippocampus. Whole transcriptome of small RNAs was performed by RNA-sequencing. We show that numerous miRNAs were induced or repressed in response to phospholipid concentrates supplementations (**Supplementary Table S1**). Compared to the control group, BMFC differentially modulated the expression of 23 miRNAs (**Figure 4A**), three up-regulated and 20 down-regulated, whereas krill oil did not exert any significant effects (**Figure 4B**). Interestingly, the mixture of both lipids dramatically modulated the expression of

these and other novel miRNAs. Namely, 119 miRNAs were modulated: 55 of them overexpressed and 64 repressed (**Figure 4C**); 18 miRNAs are shared between BMFC and BMFC + KOC (**Figure 4D**).

In order to confirm our results, we selected some miRNAs and validated them using RT-qPCR. Fifteen miRNAs were selected based on criteria such as the level of change in expression and their involvement in processes of the nervous system (**Supplementary Table S2**). Validation of the selected miRNAs of both treatments indicated that BMFC and BMFC+KOC modulate the expression of miRNAs in the hippocampus (**Figure 4E**).

Validation confirmed that both BMFC and BMFC+KOC supplementation modulated the expression of 11 miRNAs in the hippocampus: miR-99a-5p, -128-3p, -148a- 3p, -379-5p, -381-3p, -146a-5p, -30e-3p, -370-3p, -106b-3p, -770-3p and let-7f-5p (**Figure 4E**). On the other hand, miR-191a-5p and -29a-3p showed significant changes only in rats supplemented with BMFC; while miR-195-3p and -148a-5p were the only miRNAs significantly affected in the combined-supplement group. Moreover, miR-148a-3p, -370-3p, -379-5p, -99a-5p and let-7f-5p showed a significant expression increase in KOC-consuming rats, although no significant changes had been found by mass sequencing. In addition, miR-128-3p and -30e-3p were up-regulated both in the BMFC group and in the combined-supplement fed group.

With regards to the significant miRNAs validated by RT-qPCR, functional analysis of their predicted target genes showed an association with nervous system related processes, such as axon guidance, neurotrophin signaling, neuroactive ligand-receptor interaction, neuron differentiation and migration or neurological development, among others (**Supplementary Table S3**).

Supplementation with phospholipid concentrates modulates hippocampus gene expression

To appraise the diet-modulated hippocampal transcriptome, we performed gene expression microarrays. The complete results are shown in **Supplementary Table S4**. The Heat Map representation of each of the three supplemented groups is shown in **Figure 5A** and dispersion representation in **Figures 5B**, **C** and **D**.

Thirty-eight genes were found to be differentially expressed in the KOC supplemented group, 11 up-regulated and 27 down-regulated. In the group supplemented with BMFC, we found 58 differentially expressed genes (DEG), two of which with higher expression and 56 with lower expression than in the control group. Finally, the group that consumed the BMFC + KOC diet had the highest number (72) of DEG, of which 11 were up-regulated and 61 down-regulated. Some DEG were shared by two or three groups (**Figure 5E**).

Functional analysis of the differently expressed genes found in the microarray was based on different databases and displayed several neural system-related processes, among others (**Supplementary Table S5**). In all supplemented groups, we found processes related to lipid metabolism, like fatty acids synthesis and arachidonic acid metabolism, and neurotransmitter biosynthesis, such as serotonin, adrenaline and noradrenaline, transport and brain development.

BMFC-modulated genes in the hippocampus are influenced by miRNA-gene interaction.

Due to the post-transcriptional regulation by miRNAs, we performed an interaction analysis between DEG identified in the array and the miRNAs selected for validation

(**Supplementary Table S6**). This analysis related the modulated miRNAs and their targets, considering their regulatory function, i.e. up-regulated miRNAs with down-regulated targets or vice versa. In the KOC group, five miRNAs formed a network of interaction with 11 DEG (**Figure 6A**). In the BMFC group, six miRNAs showed an interaction network with eight DEG (**Figure 6B**). Finally, in the BMFC + KOC group, 32 genes were found to be potential targets of 10 miRNAs (**Figure 6C**). According to gene ontology analysis (**Figure 6D**), DEG found in the group consuming KOC are primarily involved in receptor-ligand neuroactive processes, although an involvement in transport processes such as secretion and absorption was also identified. Neuroactive receptor-ligand processes were also the main route found in the BMFC group, together with axonal orientation and Alzheimer's disease-related processes. As for the BMFC + KOC group, DEG were associated with lysosomes - cells' digestive system, and mRNA surveillance pathways, a quality control mechanism to detect and degrade abnormal mRNAs. DEG found in this group were also involved in transport processes such as secretion and absorption.

Ten DEG were then selected, based on their relation to cognitive function or neurodegenerative disease and that they were targets of miRNAs previously associated to some type of cognitive function, for validation by RT-qPCR (**Figure 6E**). In the group that consumed the KOC-supplemented diet, genes *Arc*, *Mrps23* and *Capn7* showed a statistically significant increase in expression compared to the control group. In accordance with the microarray results, *Capn7* and *Cbr3* were found to be significantly up- and down-regulated, respectively, in the BMFC group, but in this case statistical significance was not reached. Three up-regulated genes - *Tshz3, Satb2* and *Capn7*- were validated in the BMFC+KOC group with confirmation of statistically significant changes compared to the control group.

Discussion

We supplemented aged rats with a MFGM-rich concentrate and/or a krill oil concentrate for three months and report the transcriptomic and miRNA expression in their hippocampus, in addition to changes in total ceramide concentrations.

Previous works have shown that MFGM supplementation of human infants (6-11 months of age) could reduce diarrhea episodes [15] or acute otitis media infections [16], which might be related to oral microbiome changes [17]. MFGM have also shown to reduce differences in cognitive development [18] and serum lipid status [19] between formula- and breast-fed infants (from <2 until 6 months of age) when supplemented in fed-formula. Interestingly, young piglets supplemented with MFGM and other prebiotics seemed to be more advanced neurodevelopmentally [20]. In adults (18-65 y of age), buttermilk supplementation reduced cholesterol concentrations [21] and blood pressure in normotensive individuals [22]. Indeed, supplementation of overweight adults with milk fat enclosed in MFGM prevented the increase of plasma lipids compared to that of milk fat without MFGM [23]. Regarding other physiological contexts, consumption of MFGM (1g/day during 10 weeks) plus exercise have been shown to improve physical performance [24] or skeletal muscle strength (1g/day during 4 weeks) [25] of healthy adults. In the elderly, dietary supplementation with MFGM (1g/day during 3 months) combined with exercise improved the frailty status as compared to the MFGM alone [26]. Moreover, also in elderly (60-73 y), supplementation with MFGM (1g/day for 10 weeks) was shown to increase physical performance and muscle function when coupled with a light exercise program [27]. However, very few studies focused on brain function [18,20] and none on the aged brain.

We did not observe any appreciable adverse effect due to dietary supplementation. Although MFGM consumption by healthy adults has been shown to be safe at a doses of 6.5 g for 4 weeks [28], a higher rate of eczema was observed in infants administered MFGM-enriched infant formula supplementation (up to 4 months of age) as compared with standard infant formula [29].

To the best of our knowledge, our study is the first to evaluate miRNA expression in response to BFMC supplementation. Dietary modulation of miRNAs, i.e. through food bioactive compounds [30] or dietary lipids [31,32], is feasible and might be an alternative to the pharmacological modulation of miRNAs. Through gene interaction analyses, we report that miRNAs might contribute to the regulation of the modulated hippocampal genes. Gene Ontology analysis suggests that modulated genes participate in pathways related to neuroactive receptor-ligand processes, axonal orientation, Alzheimer's disease-related processes, or lysosomes pathways, most of which are related to brain function. Some such modulated genes have been previously associated with synaptic activity. Arc protein have been related to synaptic plasticity and cognition, as its neuronal activity-induced expression is critical for long-term potentiation and depression of synaptic transmission, homeostatic synaptic scaling, and adaptive functions such as long-term memory formation [33]. In this context, the significant increase of Arc observed in the KOC supplemented group could be beneficial. Although the physiological importance of Capn7, which encodes for calpain 7, is not clear, calpains are ubiquitous calcium sensitive proteases involved in essential neuronal functions including maintaining synaptic plasticity, protein turnover and cell signaling [34]. Here, the increase in the expression of this gene seen in both KOC and BMFC+KOC supplemented groups could be linked to improved synaptic plasticity. We also observed a rise in Tshz3 expression in the BMFC+KOC supplemented group.

Tshz3 deletion affects the cortical expression of a number of genes related to autism spectrum disorder (ASD) and induces ASD-relevant deficits that are associated with functional changes at synapses formed by deep-layer cerebral cortical projection neurons (CPN) [35]. Finally, Satb2 is necessary for long-term memory formation and hippocampal late-long-term potentiation and determines the expression of protein-coding genes and miRNAs linked to learning and memory in the hippocampus [36]. In this context, the increased expression of this gene seen in the BMFC+KOC supplemented group could be beneficial for well-balanced neurocognitive processes.

Even though whether the modulated genes are only a direct consequence of the modulated miRNAs it is still unclear, we show that BFMC + KOC supplementation influences the expression of several mRNAs and miRNAs in the hippocampus. In this sense, a general association with nervous system related processes, such as axon guidance, neurotrophin signaling, neuroactive ligand-receptor interaction, neuron differentiation and migration or neurological development, was found in the functional analysis of the RT-qPCR-validated miRNAs. Together with the above-mentioned genes, several miRNAs described herein have been previously related to neurodegenerative diseases. For example, it has been proposed that a loss of specific miRNAs, such as the cluster miR-29a/b-1, can contribute to increased BACE1 and Aβ levels in sporadic Alzheimer's disease [37]. If this is the case, then the increase in miR-29a expression seen in the BFMC-supplemented group could be advantageous against AD, nonetheless additional investigation is required. On the other hand, Lukiw et al. suggested that miRNA-146a-mediated modulation of complement factor H (an important repressor of the inflammatory response of the brain) gene expression may in part regulate an inflammatory response in AD brain and in stressed HN cell models of AD [38]. In this context, the rise in miR-146a expression seen in the BFMC group could be related to

changes in inflammatory processes; again, further ad-hoc research is needed. Also, Kim and colleagues identified miR-106b as a regulator of Aβ metabolism, increasing Aβ production and preventing its clearance. In this case, the rise in miR-106 observed for the BMFC+KOC supplemented group does not appear to be desirable, but literature is scant and additional studies are indispensable. We acknowledge that additional investigation is necessary. Moreover, despite the modulation of the aforementioned miRNAs, rises in expression levels could not be surely correlated with a decrease in their respective predicted target genes validated here and causality could not be established. In this context, gene expression analyses reveal changes in mRNA levels even though miRNAs can bind to mRNAs without necessarily inducing degradation but still being able to prevent protein synthesis, which could have an important impact on cell function. In fact, we cannot discard other interactions, i.e. the case of a miRNA that impacts on the protein expression levels without any apparent changes on its mRNA target, due to the solely evaluation of the transcriptome (and not protein levels). Overall, we believe that our results warrant further investigation to determine the impact of these supplements on cognitive symptoms associated with aging, that is to say more in-depth biological/molecular analysis and behavioral tests are justified.

Consumption of omega-3 fatty acids from marine sources [39] or their derivatives [40] decreases plasma triglyceride levels. Namely, krill oil consumption decreases triglyceride concentrations [41] both in healthy [42] and hypertrygliceridemic subjects [43]. The bioavailability of n-3 FAs from krill has been shown to be similar to that of other long-chain n-3 polyunsaturated fatty acids [44]; however, their specific effects in the elderly have not been studied thus far. A recent study suggested that dietary krill oil supplementation enhanced neurocognitive function in aging mice, by changing the expression of Celsr3 and Ppp1r1b [45], which are implicated in memory and learning

process. In this study, we did not observe any change in triglyceride concentrations in supplemented aged rats. Whether krill oil or other sources of omega-3 fatty acids also influence lipid levels in the elderly remains unresolved. Indeed, omega-3 fatty acid-rich oils (beginning at 12 months of age) apparently shorten the life span of long-lived F1 mice and krill oil modestly increases bilirubin, triglyceride, and glucose levels [46]. Whether the lowering effects of omega-3 fatty acid-rich oils on triglyceride levels are reduced by aging is unknown.

We also report that dietary supplementation with different phospholipids did not remarkably influence the lipid profile of the hippocampus, with the exception for a significant reduction in ceramide levels in rats supplemented with a MFGM-rich concentrate in combination with a krill oil concentrate. Whether changes in ceramide levels can contribute to delaying mild-cognitive decline due to aging is not clear but further investigation is entitled. Ceramides play an important role in the neurons and its composition changes through the different stages of development [47]. Indeed, ceramide accumulations in the brain results in a deregulation of energy balance and lead to different disorders as obesity, disturbance of glucose homeostasis [48], neurodegenerative diseases [49] and AD pathogenesis [50,51]. Our data shows changes in the transcriptomic and miRNA expression profiling in response to dietary supplementation with a MFGM-rich concentrate in the hippocampus. Whether there is a direct causality between these changes and the reduction in ceramide levels is unknown, but, to the best of our knowledge, cannot be discarded. Ceramide deregulation has been shown to cause distinct global alterations of gene expression in hepatocyte cell lines [52] or changes in miRNA expression in multiple myeloma cells *in vitro* [53]. However, the *in vivo* effects of these changes on the whole transcriptome or miRNome are understudied, especially in the hippocampus.

In summary, the experimental evidence presented here and elsewhere suggests that a buttermilk fat concentrate, rich in MFGM, alone or in combination with a krill oil concentrate, may influence cognitive development [18] or brain function [20] by targeting both genetic and epigenetic mechanisms through miRNA modulation. Whether these epigenomic and nutrigenomic changes can influence functional readouts is still unknown. As mentioned in the Introduction, the increasing incidence of age-induced cognitive declines calls for immediate action and the use of dietary supplements, e.g. BFMC + KOC and others warrants intense investigation.

Material and methods

Animals, diets and experimental design

We followed the Guide to the Care and Use of Laboratory Animals, published by the US National Research Council (Eight Edition, 2010). The animal experimentation committee of the National University of Distance Education (UNED) approved these experiments. Nine-month old Wistar rats (n=46) were purchased from Charles River Laboratories (Barcelona, Spain) and were housed for nine months. At 18 months of age (when rats begin to present a variety of cognitive symptoms associated with aging), the animals were randomly assigned to four experimental groups of diet supplementation with phospho- and sphingolipids concentrates from buttermilk (BM) and krill oil (KO), or a combination of both (**Table 1**). Concentrates were produced at the Institute of Food Science Research (CIAL, Madrid, Spain) and were given in the form of frozen strawberry jellies. Nutritional and methyl ester fatty acids composition of the concentrates are described in **Table 2** and **Table 3**, respectively. The nutritional intervention lasted for three months. Briefly, rats were weighed every week during the supplementation period, after which they were sacrificed by decapitation. Blood

samples were centrifuged for plasma collection at 1500 xg for 15 minutes, RT. Tissues and organs were quickly extracted and frozen in liquid nitrogen. All samples were stored at -80 ˚C.

Determination of plasma lipids and lipoproteins

Plasma concentrations of cholesterol and triglycerides were analyzed enzymatically using commercial kits (Centronic, Germany & Bradford Diagnostics, Kemia Científica S.A., Spain). To determine the plasma lipoprotein profile, a pool of plasmas was subjected to fast protein liquid chromatography (FPLC) gel filtration through a Superose 6 HR 10/30 column (Pharmacia). Samples (220 µl) were eluted with 150 mM NaCl, 10 mM Tris–HCl, 2 mM Na2-EDTA and 0.02% NaN3, pH 7.4, at a flow rate of 0.3 ml/min, and 0.4-ml fractions were collected.

Cholesterol, triglyceride, non-esterified fatty acid (FAs) and phospholipid levels were determined in plasma samples using appropriate commercial assay kits, according to the instruction's manual (Spinreact, Sant Esteve de Bas, Spain & Wako Chemicals GmbH, Neuss, Germany).

Ceramide and lipid analysis

Tissue lipids were extracted using the Folch method described by Löfgren *et al.* [54] with slight modifications. Briefly, tissues samples were dissolved in methanol in 50 mL glass tubes. The mix was sonicated in an ultrasonic processor (Dr. Hielscher, Teltow, Germany) during 15 second, two cycles. Then, dichloromethane (1:2 methanol/dichloromethane) was added and mixed during 20 min. Acetic acid 20 mM (1:3 acetic acid/dichloromethane) was added and samples were again mixed for 20 min. Samples were centrifuged at 2100 rpm, 5 min, 4 ºC. Bottom organic phases were transferred to a new glass tube and the methanolic phase was washed with dichloromethane (1:1 dichloromethane/ methanol) and mixed for 10 min before

centrifugation with the same conditions as described above. The organic phases were collected and mixed and filtered through 0.45 µm, evaporated with nitrogen and weighted. Lipids extracts were maintained at -35 ºC. The separation of ceramides of lipid extracts from tissues samples were performed with a HPLC Agilent Technologies, model 1200 (Agilent Technologies, Palo Alto, CA, USA) coupled to an evaporative light scattering detector (ELSD) (SEDERE. SEDEX 85 model, Alfortville Cedex, France) using pre-filtered compressed air as the nebulizing gas at pressure of 350 KPa, temperature of 90ºC and the gain was set at 6. Two columns Zorvax Rx-SIL column (Agilent Technologies, Palo Alto, CA, USA) of 250 mm × 4.5 mm and 5 µm particle size, were coupled in series with a precolumn with the same refill. They were equilibrated at 40 ºC. The injection volume was 50 µL at concentration of 5 mg/mL in CH_2Cl_2 using chromatographic solvent gradient as we have previously described [55].

RNA isolation

Total small and large RNA extraction from hippocampus samples were performed according to the manufacturer's instructions (NucleoSpin® miRNA kit, Macherey Nagel, Düren, Germany). RNA quantity was determined by NanoDrop® ND-2000 Spectrophotometer (Thermo Fisher Scientific Inc., Spain).

Small RNA library construction and sequencing

Purity and integrity of RNA extracted from hippocampal samples (n = 5 per group), were assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). A NEBNext® Small RNA Library Prep Set for Illumina (New England BioLabs, Ipswich, MA) was used to prepare the libraries according to the manufacturer's protocol. The cDNA library was sequenced with NextSeq 500 from Illumina. To

facilitate the analysis a filter of sequences with a maximum length of 50NT was applied. Samples passed the quality controls performed through the FASTQC tool. Bowtie2 was used for sequence alignment with reference genomes, first against rat miRNA sequences downloaded from miRbase, and then against the rat genome Rnor_5.0 (Ensembl release 79). The integrated Genome Viewer (IGV) software was used for alignment visualization. Finally, gene counting was carried out using HTSeq-count to allow differential expression analysis, mainly, of the miRNAs obtained from each experimental group versus the control group.

Arrays

Expression arrays were performed in hippocampal tissue of three samples per study group, using the 4x44K complete rat genome platform from Affymetrix (Clariom S Assays). Each microarray screens approximately 22,900 unique rat genes and transcripts. Briefly, double-stranded cDNA was synthesized from total RNA using One-cycle cDNA Synthesis Kit (Affymetrix, Santa Clara, CA). cRNA was fragmented and hybridized to the Affymetrix matrix following the manufacturer's instructions. Finally, microarrays were washed and cartridges were scanned in a GeneChip® 3000 scanner for fluorescence signal acquisition.

The generated output files in CEL format were used for downstream analysis. Data was normalized with the RMA method. Probes with lower variance were filtered out, resulting in approximately 12000 remaining probes. Differential gene expression was assessed with the Bioconductor Limma package. Genes with a FDR lower than 0.1 were considered as statistically significant.

Interactions analysis

For interaction analysis, we screened miRNAs selected for validation. Prediction of miRNA targets was obtained using the Diana microT-CDS algorithm. Only the corresponding targets that showed statistically significant differential gene expression in microarrays were considered as valid interactions. Target point sizes are directly correlated with the number of interactions within the set of miRNAs.

Functional analysis and miRNA target prediction

Genecodis3 algorithm was used for functional analysis of the obtained gene lists, focusing on biological processes and pathways, using the Gene Ontology and KEGG pathway databases respectively. Predicted miRNA gene targets were obtained using the Diana microT-CDS algorithm. For each miRNA, the prediction score was set to a convenient value in order to obtain in the output a gene target list ranging from 200 to 1500 items.

Validation of miRNA and gene expression by RT-qPCR

miScript®II reverse transcription kit (Qiagen, Germantown, MD) was used to converted total RNA into first strand cDNA for miRNA validations whereas iScript™ Advanced cDNA Synthesis Kit (BIO-RAD, Hercules, CA) was used for gene validations, both according to the manufacturer's guidelines.

Selected miRNAs were obtained from the miRBase database, and the genes for the validation were designed using Primer3 online program and amplify4 (Supplementary Tables S7 and S8). Designed primers were constructed by ISOGEN (Life Science, Belgium). Real time PCR was performed in a 384-plate format using a 7900HT system (Life Technologies, Alcobendas, Spain). Cycling conditions for both gene and miRNA amplification included a first step of activation at 95 °C 15 minutes. For gene amplification, 40 cycles at 94 °C for 15 seconds for the denaturation, annealing at 58 °C

for 30 seconds were applied subsequently. As for miRNA amplification, 40 cycles of denaturation at 94 °C for 15 seconds, annealing at 58 °C for 30 seconds and an extension at 70 °C for 15 seconds were applied. Finally, a dissociation curve step at 95 °C for 15 seconds, followed by 15 seconds at 60 °C and 95 °C for 15 seconds was carried out for both amplification types. miScript SYBR Green qPCR Master Mix (Qiagen,Madrid, Spain) was used for both validations. GAPDH and RNU6 were used as reference genes for gene and miRNA data normalization, respectively. The ΔΔCt method was used for gene expression analysis and fold-change values were reported as $2^{-(\Delta\Delta Ct)}$.

Statistical analysis

Significance of the functional enrichment results was assessed using the hypergeometric test and items with corrected $p < 0.05$ were considered as statistically significant. Differences between groups were assessed by one-way ANOVA followed by Bonferroni test for post hoc comparisons. $p < 0.05$ was considered as statistically significant. Results are presented as means ± SEM. Statistical analyses were performed with GraphPad Prism 7.02 software (GraphPad Software, Inc., La Jolla, CA, USA).

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Author contributions

JF, CV and FV designed the supplementation study. AD, MCC and JTC designed the miRNA/genomic workflow. JTC, MCC, AGS, EBM, DGC performed experiments. RM-H performed bioinformatic analysis. MCC, JTC, AD and FV wrote the manuscript. All authors approved the submission of the final version of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest associated with this publication.

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Figure legends

Figure 1. Variation of body mass. Values are expressed as mean ± SEM of the mean. BMFC: buttermilk fat concentrate rich in phospho- and sphingolipids; KOC: krill oil concentrate rich in omega-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid) and phospholipids; BMFC+KOC: combination of BMFC and KOC.

Figure 2. Lipid levels and lipoprotein profile of rat plasma. A) FPLC profile of plasma cholesterol and triglycerides. B) Cholesterol, triglycerides, non-esterified fatty acids, and phospholipid levels in plasma. Values are means ± SEM. Statistically significant difference from control at *p< 0.05-0.005; **p< 0.005-0.0005. BMFC: buttermilk fat concentrate rich in phospho- and sphingolipids; KOC: krill oil concentrate rich in

omega-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid) and phospholipids; BMFC+KOC: combination of BMFC and KOC.

Figure 3. Ceramide concentrations in the hippocampus. Values are the mean ± SEM, n≥10 per group. Different letters denote differences at $p< 0.05$. BMFC: buttermilk fat concentrate rich in phospho- and sphingolipids; KOC: krill oil concentrate rich in omega-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid) and phospholipids; BMFC+KOC: combination of BMFC and KOC.

Figure 4. $sRNA_{Seq}$ results. Smear plots for control vs KOC (**A**), BMFC (**C**) and KOC+BMFC (**B**) groups. **D)** Venn diagram of differentially expressed miRNAs. **E)** Validation of selected miRNAs. Values are means ± SEM. Statistically significant difference vs control at $*p< 0.05$-0.005; $**p< 0.005$-0.0005; $***p< 0.0005$. BMFC: buttermilk fat concentrate rich in phospho- and sphingolipids; KOC: krill oil concentrate rich in omega-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid) and phospholipids; BMFC+KOC: combination of BMFC and KOC.

Figure 5. Gene expression analysis. A) Heatmap of microarray data. Scattered plots for KOC (B), BMFC (C) and KOC+BMFC (D) groups. E) Venn diagram of differently expressed genes. BMFC: buttermilk fat concentrate rich in phospho- and sphingolipids; KOC: krill oil concentrate rich in omega-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid) and phospholipids; BMFC+KOC: combination of BMFC and KOC.

Figure 6. Genetic interaction analysis between miRNAs and their possible mRNAs targets for KOC (A), BMFC (B) and BMFC+KOC (C) groups. D) Functional enrichment analysis of differentially expressed genes. E) Validation of potential target genes of previously validated miRNAs. Arc: activity-regulated cytoskeleton-associated

protein; Cbr3: carbonyl reductase 3; Tshz3: teashirt zinc finger homeobox 3; Pla2g5: phospholipase A2 group V; Htr2c: 5-hydroxytryptamine receptor 2C; Mrps23: mitochondrial ribosomal protein S23; Sst: somatostatin; Satb2: SATB homeobox 2; Ip6k1: inositol hexakisphosphate kinase 1; Capn7: calpain 7. BMFC: buttermilk fat concentrate rich in phospho- and sphingolipids; KOC: krill oil concentrate rich in omega-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid) and phospholipids; BMFC+KOC: combination of BMFC and KOC.

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Table 1. Experimental design and administered diets.

	Experimental design			
Diets	***Control***	***BMFC***	***KOC***	***BMFC+KOC***
standard diet	EURodent Diet 22%	EURodent Diet 22%	EURodent Diet 22%	EURodent Diet 22%

Daily jelly	70 mg refined olive oil	70 mg BMFC	70 mg KOC	70 mg BMFC+70 mg KOC

BMFC: buttermilk fat concentrate rich in phospho- and sphingolipids; KOC: krill oil concentrate rich in omega-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid) and phospholipids; BMFC+KOC: combination of BMFC and KOC. EURodent Diet 22% (LabDiet, San Luis, Missouri).

Table 2. Nutritional composition of the supplements.

Dose contribution/ animal/Day	Control	BMFC	KOC	BMFC+KOC
Energy (Kcal)	201,07	201,11	201,07	201,75
Lipids (g)	1,82	1,82	1,82	1,89
Carbohydrates (g)	32,10	32,11	32,10	32,11
Fiber (g)	2,05	2,05	2,05	2,05
Proteins (g)	11,01	11,01	11,01	11,01

BMFC, buttermilk fat concentrate rich in phospho- and sphingolipids; KOC, krill oil concentrate rich in omega-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid) and phospholipids; BMFC+KOC, combination of BMFC and KOC.

Table 3. Fatty acids (as methyl esters) included in the nutritional supplements.

MEFA g/100g supplement	**Control**	**BMFC**	**KOC**	**BMFC+KOC**
Σ SFA	15,29 ± 0,11	47,77 ± 0,61	27,66 ± 0,27	32,90 ± 0,64
Σ MFA	77,54 ± 0,55	40,20 ± 0,72	23,08 ± 0,15	34,18 ± 1,32
Σ PUFA	6,66 ± 0,16	11,39 ± 0,42	44,73 ± 0,62	33,17 ± 0,83
Σ n-6	6,26 ± 0,10	10,13 ± 0,37	2,09 ± 0,05	5,77 ± 0,50
Σ n-3	0,41 ± 0,06	0.61 ± 0,04	43,06 ± 0,65	24,14 ± 1,07
Σ mFA	0,50 ± 0,10	1,54 ± 0,09	2,22 ± 0,22	2,68 ± 0,07

MEFA, methyl esters of fatty acids; SFA, saturated fatty acids; MFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n-6, omega−6 fatty acids; n-3, omega−3 fatty acids; mFa, other minor fatty acids; BMFC, buttermilk fat concentrate rich in phospho- and sphingolipids; KOC, krill oil concentrate rich in omega-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid) and phospholipids; BMFC+KOC, combination of BMFC and KOC.

Modulation of miRNA expression in aged rat hippocampus by buttermilk and krill oil

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1. miRNA sequencing results.

Table S2. Selected miRNAs for validation by RT-qPCR.

Table S3. Functional analysis of the significant miRNA of NGS validated by PCR. (online link)

https://www.dropbox.com/sh/itsrudehm25jgvd/AADe9jQkpxxQFys5bXeBxJgBa?dl=0

Table S4. Gene expression microarray results.

Table S5. Functional analysis of the significant genes of the array. (online link)

https://www.dropbox.com/sh/itsrudehm25jgvd/AADe9jQkpxxQFys5bXeBxJgBa?dl=0

Table S6. Differently expressed genes from microarray results selected for validation by RT-qPCR.

Table S7. Sequence of miRNAs primers selected for validation.

Table S8. Sequence of genes primers selected for validation.

Table S1. miRNA sequencing results

	miRNA	logFC	PValue	FDR
BMFC vs Control	**miR-146a-3p**	-3.674	0.000	0.013
	miR-3552	-3.389	0.000	0.013
	miR-181a-5p	-2.760	0.001	0.039
	miR-17-1-3p	-2.168	0.001	0.032
	miR-483-3p	-2.083	0.001	0.032
	miR-17-5p	-1.756	0.001	0.033
	miR-20a-5p	-1.575	0.000	0.013
	miR-339-5p	-1.569	0.000	0.013
	miR-99a-3p	-1.501	0.001	0.032
	miR-3102	-1.397	0.000	0.008
	miR-31a-5p	-1.394	0.001	0.033
	miR-582-5p	-1.376	0.001	0.032
	miR-3065-3p	-1.261	0.001	0.035
	miR-369-3p	-1.255	0.002	0.044
	miR-872-3p	-1.238	0.001	0.039
	miR-219a-5p	-1.147	0.001	0.032
	miR-674-5p	-1.004	0.002	0.041
	miR-450a-5p	-0.970	0.001	0.032
	miR-15b-5p	-0.956	0.002	0.044
	miR-181b-5p	-0.932	0.001	0.040
	let-7f-5p	0.822	0.002	0.045
	miR-148a-5p	0.918	0.002	0.047
	miR-195-3p	1.023	0.002	0.042

	miRNA	logFC	PValue	FDR
	miR-17-5p	-2.747	0.001	0.008
	miR-3583-3p	-2.575	0.011	0.045
	miR-362-3p	-2.464	0.002	0.013
	miR-17-1-3p	-2.377	0.007	0.031
	miR-20a-5p	-2.305	0.000	0.000
	miR-449a-5p	-2.287	0.002	0.014
	miR-369-3p	-2.254	0.000	0.006
	miR-362-5p	-2.242	0.001	0.009
	let-7f-2-3p	-2.192	0.002	0.015
	miR-339-5p	-2.154	0.000	0.004
	miR-764-3p	-2.115	0.011	0.043
	miR-107-3p	-2.102	0.000	0.002
	miR-194-5p	-1.922	0.000	0.002
	miR-770-5p	-1.899	0.001	0.009
	miR-144-5p	-1.896	0.006	0.028
	miR-93-3p	-1.876	0.003	0.018
	miR-374-5p	-1.849	0.000	0.001
	miR-582-5p	-1.824	0.000	0.003
	miR-3065-3p	-1.797	0.000	0.002
	miR-137-3p	-1.771	0.000	0.002
	miR-99a-3p	-1.699	0.001	0.008
	miR-106b-5p	-1.696	0.000	0.001
	miR-324-3p	-1.689	0.000	0.005
	miR-539-3p	-1.671	0.000	0.006
	miR-143-5p	-1.592	0.001	0.008
	miR-34c-3p	-1.585	0.003	0.017
	miR-30c-5p	-1.546	0.001	0.007
	miR-494-3p	-1.543	0.000	0.006
	miR-872-3p	-1.522	0.001	0.009

BMFC+KOC vs Control	**miR-98-3p**	-1.465	0.009	0.038
	miR-31a-5p	-1.457	0.000	0.004
	miR-16-5p	-1.453	0.000	0.002
	miR-126a-5p	-1.408	0.001	0.008
	miR-708-5p	-1.396	0.000	0.005
	miR-410-3p	-1.391	0.000	0.006
	miR-1193-3p	-1.344	0.008	0.033
	miR-425-3p	-1.326	0.002	0.015
	miR-93-5p	-1.272	0.000	0.004
	miR-674-5p	-1.266	0.001	0.008
	miR-376b-3p	-1.238	0.004	0.022
	miR-361-5p	-1.225	0.001	0.009
	miR-181b-5p	-1.222	0.001	0.009
	miR-221-3p	-1.216	0.001	0.008
	miR-219a-5p	-1.200	0.007	0.033
	miR-450a-5p	-1.181	0.001	0.008
	miR-186-5p	-1.177	0.000	0.006
	miR-138-5p	-1.166	0.001	0.009
	miR-497-5p	-1.146	0.007	0.029
	miR-3589	-1.126	0.003	0.019
	let-7e-3p	-1.079	0.001	0.009
	miR-384-5p	-1.073	0.004	0.022
	miR-98-5p	-1.071	0.001	0.009
	miR-15b-5p	-1.048	0.006	0.027
	miR-384-3p	-0.997	0.006	0.028
	miR-874-3p	-0.986	0.004	0.023
	miR-379-3p	-0.970	0.002	0.015
	miR-23a-3p	-0.892	0.006	0.028
	miR-195-5p	-0.891	0.006	0.028
	miR-191a-5p	-0.874	0.003	0.016
	miR-140-5p	-0.863	0.003	0.019
	miR-421-3p	-0.863	0.009	0.038
	miR-26b-5p	-0.848	0.004	0.022
	miR-146a-5p	-0.844	0.004	0.021
	miR-29a-3p	-0.835	0.007	0.033
	miR-543-3p	0.690	0.012	0.047
	miR-455-5p	0.763	0.012	0.047
	miR-495	0.810	0.006	0.028
	let-7i-5p	0.811	0.007	0.030
	miR-541-5p	0.822	0.009	0.038
	miR-134-5p	0.840	0.010	0.041
	miR-143-3p	0.884	0.005	0.024
	miR-135a-3p	0.899	0.008	0.033
	miR-125-1-3p	0.899	0.009	0.038
	let-7f-5p	0.905	0.005	0.027
	miR-139-5p	0.923	0.001	0.009
	miR-99a-5p	0.940	0.004	0.021
	miR-361-3p	0.965	0.006	0.028
	miR-185-3p	0.969	0.009	0.038
	miR-99b-5p	0.973	0.010	0.038
	miR-380-5p	0.975	0.005	0.023
	miR-6331	0.976	0.004	0.021
	miR-128-3p	0.981	0.003	0.018
	miR-30c-2-3p	0.995	0.001	0.010
	miR-1843-5p	1.008	0.005	0.023
	miR-106b-3p	1.015	0.003	0.018
	miR-192-5p	1.042	0.001	0.010
	miR-3559-3p	1.060	0.009	0.038

	miR-1843-5p	1.092	0.004	0.022
	miR-99b-3p	1.156	0.001	0.008
	miR-184	1.180	0.003	0.019
	miR-30e-3p	1.208	0.002	0.013
	miR-676	1.227	0.000	0.003
	miR-344-1-3p	1.236	0.000	0.001
	miR-673-3p	1.264	0.000	0.004
	miR-770-3p	1.302	0.000	0.005
	miR-493-3p	1.320	0.002	0.015
	miR-370-3p	1.367	0.001	0.009
	miR-485-5p	1.383	0.001	0.010
	miR-379-5p	1.393	0.000	0.002
	miR-3557-5p	1.422	0.000	0.003
	miR-127-3p	1.423	0.000	0.001
	miR-129-5p	1.460	0.002	0.015
	miR-219-1-3p	1.464	0.002	0.012
	miR-150-3p	1.605	0.001	0.007
	miR-671	1.615	0.000	0.004
	miR-381-3p	1.639	0.000	0.002
	miR-674-3p	1.665	0.000	0.001
	miR-148a-5p	1.682	0.000	0.001
	miR-6329	1.690	0.000	0.001
	miR-10a-5p	1.697	0.002	0.012
	miR-27a-5p	1.725	0.000	0.002
	miR-409b	1.728	0.000	0.001
	miR-10b-5p	1.748	0.002	0.012
	miR-195-3p	1.829	0.000	0.001
	miR-708-3p	1.935	0.000	0.000
	miR-6315	1.984	0.000	0.003
	miR-151-3p	2.087	0.000	0.004
	miR-148a-3p	2.288	0.000	0.000
	miR-124-5p	3.505	0.002	0.013

BMFC: buttermilk fat concentrate rich in phospho- and sphingolipids; KOC: krill oil concentrate rich in omega-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid) and phospholipids; BMFC+KOC: combination of BMFC and KOC.

Table S2. Selected miRNAs for validation by RT-qPCR.

	miRNA	logFC	PValue	FDR	Function
BMFC	**let-7f-5p**	0.822	0.002	0.045	Down-regulation in AD
	miR-195-3p	1.023	0.002	0.042	Role in determining dementia susceptibility
	miR-148a-5p	0.918	0.002	0.047	Inhibition induces hepatocellular tumorigenesis
BMFC+KOC	**miR-99a-5p**	0.940	0.004	0.021	Neuroprotective effect
	miR-128-3p	0.981	0.003	0.018	Critical for hippocampus-related contextual learning
	miR-148a-3p	2.288	0.000	0.000	Downregulation in AD
	miR-29a-3p	-0.835	0.007	0.033	Neuronal synapse formation and plasticity
	miR-191a-5p	-0.874	0.003	0.016	Target genes to apoptosis and cell death pathways
	miR-381-3p	1.639	0.000	0.002	Linked to insulin resistance or T2D
	miR-379-5p	1.393	0.000	0.002	Predispose to neuropathic pain identified
	miR-30e-3p	1.208	0.002	0.013	Linked to insulin resistance
	miR-146a-5p	-0.844	0.004	0.021	Associated with AD.
	miR-370-3p	1.367	0.001	0.009	Downregulated by Mild Stress in Rat Hippocampal Tissues
	let-7f-5p	0.905	0.005	0.027	Downregulation in AD
	miR-770-3p	1.302	0.000	0.005	Down-regulated in the hippocampus of Sprague-Dawley rats with temporal lobe epilepsy
	miR-106b-3p	1.015	0.003	0.018	Protect mitochondrial functions
	miR-195-3p	1.829	0.000	0.001	Downregulation in AD
	miR-148a-5p	1.682	0.000	0.001	Cancer

BMFC: buttermilk fat concentrate rich in phospho- and sphingolipids; KOC: krill oil concentrate rich in omega-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid) and phospholipids; BMFC+KOC: combination of BMFC and KOC.

Table S3 (see online file)

Table S4. Gene expression microarray results.

	Gene	logFC	P.Value	Adj.P.Val
KOC	**Kcnj13**	-3	0.0000	0.0315
	Dpt	-2.4	0.0000	0.0275
	Tph1	-2.1	0.0000	0.0000
	Slc35f4	-2	0.0001	0.0452
	Exoc5	-1.8	0.0000	0.0315
	Barx1	-1.7	0.0000	0.0005
	Ippk	-1.5	0.0000	0.0013
	Lilrb3l	-1.4	0.0000	0.0013
	P4hb	-1.3	0.0000	0.0008
	Cenpn	-1.3	0.0001	0.0438
	---	-1.2	0.0000	0.0049
	Enpp2	-1.2	0.0001	0.0380
	Osr1	-1.1	0.0000	0.0275
	Ptgds	-1.1	0.0000	0.0315
	LOC654482	-1.1	0.0001	0.0341
	Slc6a20	-1.1	0.0001	0.0380
	Bicd2	-1	0.0000	0.0226
	Ch25h	-1	0.0000	0.0293
	Arhgef15	-1	0.0001	0.0371
	Sox21	-1	0.0001	0.0452
	Nutm2f	-1	0.0001	0.0452
	Rarres1	-0.9	0.0000	0.0315
	Car13	-0.9	0.0001	0.0332
	Id4	-0.9	0.0001	0.0438
	Efhc1	-0.9	0.0002	0.0490
	Ctsc	-0.8	0.0001	0.0452
	Aox4	-0.8	0.0001	0.0452
	Gpr135	0.8	0.0001	0.0426
	Pygo2	0.8	0.0001	0.0452
	Fam163b	0.8	0.0001	0.0452
	Ptpn4	0.8	0.0001	0.0452
	St6gal2	0.8	0.0002	0.0495
	Pak6	0.9	0.0000	0.0315
	Arc	0.9	0.0000	0.0315
	Ramp3	0.9	0.0001	0.0341
	Cckbr	0.9	0.0001	0.0371
	N4bp2	1	0.0000	0.0266
	Yeats2	1	0.0001	0.0438
BMFC	**Kcnj13**	-2.8	2.53E-12	2.91E-08
	Dpt	-2.3	8.23E-11	4.74E-07
	Zfp358	-2.1	4.15E-09	6.81E-06
	Cbr3	-2	2.60E-09	4.99E-06

	Mfrp	-2	1.51E-08	2.17E-05
	Tmem72	-1.9	5.70E-08	7.29E-05
	Sostdc1	-1.8	4.66E-10	1.34E-06
	Barx1	-1.8	4.56E-10	1.34E-06
	Ippk	-1.6	2.29E-09	4.99E-06
	Slco1a5	-1.6	1.05E-07	0.00011518
	Tph1	-1.6	2.45E-06	0.0011723
	Ahsg	-1.5	3.48E-07	0.00026687
	Slc35f4	-1.4	1.10E-07	0.00011518
	Exoc5	-1.4	1.75E-07	0.00016792
	Slc4a5	-1.4	2.40E-07	0.00020184
	Slc13a4	-1.4	4.28E-07	0.00029527
	Prlr	-1.4	1.45E-05	0.00476127
	Ict1	-1.3	2.46E-07	0.00020184
	C7	-1.3	4.88E-07	0.00029527
	Prr32	-1.3	6.79E-07	0.00039051
	Zfp24	-1.3	6.74E-06	0.0025839
	Ptgds	-1.2	4.47E-07	0.00029527
	---	-1.2	4.70E-07	0.00029527
	Slc6a20	-1.2	7.25E-07	0.00039735
	Aqp1	-1.2	7.68E-06	0.00276169
	Cldn2	-1.2	5.98E-05	0.01465844
	Myog	-1.1	1.60E-06	0.00079918
	Ctsc	-1.1	3.03E-06	0.00134359
	Enpp2	-1.1	3.04E-06	0.00134359
	Ch25h	-1.1	3.43E-06	0.00146244
	Abca4	-1.1	4.89E-06	0.00200889
	Folr1	-1.1	2.45E-05	0.00721838
	Cbr1	-1.1	5.20E-05	0.01329863
	Emp3	-1	6.23E-06	0.00247225
	Bicd2	-1	1.24E-05	0.00430572
	LOC654482	-1	1.31E-05	0.0044311
	Mospd1	-1	1.67E-05	0.00533843
	Lepr	-1	1.77E-05	0.00551727
	P4hb	-1	2.09E-05	0.00632722
	Atp11c	-1	3.81E-05	0.01086025
	Cenpn	-1	4.38E-05	0.01184456
	Igf2	-1	6.44E-05	0.01544012
	Rpl9	-1	8.19E-05	0.01884002
	Sst	-1	8.66E-05	0.01952348
	Tmem27	-1	0.000134	0.02854155
	Elovl7	-0.9	3.87E-05	0.01086025
	Pla2g5	-0.9	4.43E-05	0.01184456
	Ranbp3l	-0.9	4.77E-05	0.01247554
	Hmgb2l1	-0.9	5.99E-05	0.01465844

	Trim34	-0.9	6.94E-05	0.01628543
	Capn7	-0.9	0.00011415	0.02525007
	Slc2a12	-0.9	0.00013173	0.02854155
	C1qtnf3	-0.9	0.00014208	0.02971226
	Mdk	-0.9	0.00014941	0.03068729
	Slc22a8	-0.8	0.00021362	0.04310616
	Spata20	-0.8	0.00022686	0.04498921
	Arc	1	7.18E-06	0.00266579
	Adss	1.3	1.03E-06	0.00053942
BMFC+KOC	**Zfp358**	-3.6	0.0000	0.0000
	Kcnj13	-3.9	0.0000	0.0000
	Dpt	-3.7	0.0000	0.0000
	Zfp24	-3.5	0.0000	0.0000
	Cbr3	-3.4	0.0000	0.0000
	Mfrp	-3.2	0.0000	0.0000
	Sostdc1	-2.9	0.0000	0.0000
	Tmem72	-2.9	0.0000	0.0000
	Prlr	-2.6	0.0000	0.0000
	Ahsg	-2.4	0.0000	0.0000
	Exoc5	-2.4	0.0000	0.0000
	Cldn2	-2.4	0.0000	0.0001
	Slc35f4	-2.2	0.0000	0.0000
	Slc4a5	-2.1	0.0000	0.0000
	Folr1	-2	0.0000	0.0000
	Prr32	-2	0.0000	0.0000
	Slco1a5	-2	0.0000	0.0000
	Ict1	-1.9	0.0000	0.0000
	Tmem27	-1.8	0.0000	0.0010
	Sst	-1.6	0.0000	0.0011
	Cenpn	-1.5	0.0000	0.0002
	Aqp1	-1.5	0.0000	0.0007
	Abca4	-1.5	0.0000	0.0007
	Cbr1	-1.5	0.0000	0.0022
	Slc13a4	-1.5	0.0000	0.0117
	Htr2c	-1.4	0.0000	0.0008
	Capn7	-1.3	0.0000	0.0014
	Itpripl1	-1.2	0.0000	0.0015
	Glycam1	-1.2	0.0000	0.0015
	Pla2g5	-1.2	0.0000	0.0017
	LOC654482	-1.2	0.0000	0.0022
	Rpl9	-1.2	0.0000	0.0063
	Enpp2	-1.1	0.0000	0.0021
	Spata20	-1.1	0.0000	0.0044
	Itgb6	-1.1	0.0000	0.0057
	Atp11c	-1.1	0.0000	0.0070

	Scgb1c1	-1.1	0.0000	0.0077
	Aimp2	-1.1	0.0000	0.0098
	Mdk	-1.1	0.0001	0.0231
	Slc2a12	-1.1	0.0003	0.0475
	Efhc1	-1	0.0000	0.0075
	Tbc1d10a	-1	0.0000	0.0088
	Creg1	-1	0.0001	0.0177
	Diaph3	-1	0.0001	0.0239
	Narfl	-0.9	0.0000	0.0082
	Wdr63	-0.9	0.0000	0.0100
	RGD1564074	-0.9	0.0000	0.0111
	Glipr2	-0.9	0.0000	0.0117
	Fbxo45	-0.9	0.0000	0.0119
	LOC100909960	-0.9	0.0001	0.0134
	Lama2	-0.9	0.0001	0.0163
	Cars2	-0.9	0.0001	0.0264
	Olr109	-0.9	0.0001	0.0289
	Slc4a2	-0.9	0.0002	0.0316
	Wfs1	-0.9	0.0002	0.0365
	Muc19l1	-0.8	0.0001	0.0239
	Igfbpl1	-0.8	0.0002	0.0383
	Mast3	-0.8	0.0002	0.0384
	---	-0.8	0.0002	0.0415
	Fam92b	-0.8	0.0003	0.0473
	Six3	-0.8	0.0003	0.0486
	LOC100911887	0.8	0.0001	0.0276
	Samd15	0.8	0.0002	0.0365
	Tnks	0.8	0.0003	0.0465
	Sptssb	0.8	0.0003	0.0469
	Satb2	0.8	0.0003	0.0489
	ATP6	0.8	0.0003	0.0489
	MGC116197	0.9	0.0002	0.0342
	LOC102550585	1	0.0000	0.0068
	Cckbr	1	0.0000	0.0070
	LOC102555217	1	0.0000	0.0082
	Npas4	1	0.0002	0.0351

BMFC: buttermilk fat concentrate rich in phospho- and sphingolipids; KOC: krill oil concentrate rich in omega-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid) and phospholipids; BMFC+KOC: combination of BMFC and KOC.

Table S5 (see online file)

Table S6. Differently expressed genes from microarray results selected for validation by RT-qPCR.

Gene	miR Interaction	Study group	Gene Function	miRNA Function
Arc	7f-5p	KOC	Neuronal plasticity and memory	Downregulation in AD
Cbr3	191a-5p	BMFC	Parkinson's disease	Target genes to apoptosis and cell death pathways
	106b	BMFC+KOC	Parkinson's disease.	Protect mitochondrial functions
tshz3	148a-5p	BMFC+KOC	Reduced expression of this gene and consequent caspase upregulation may be correlated with progression of Alzheimer's disease	Inhibition induces hepatocellular tumorigenesis
	195-3p			Downregulation in AD
	30e-3p			Linked to insulin resistance
	381-3p			
pla2g5	106b	BMFC+KOC	Increased in several neurological and neurodegenerative disorders	Protect mitochondrial functions
	148a-3p			Downregulation in AD
	195-3p			Downregulation in AD
htr2c	195-3p	BMFC+KOC	Signaling of neurotransmitters	Downregulation in AD
	379-5p			Predispose to neuropathic pain identified
Mrps23	379-5p	KOC	Mitochondrial ribosomal proteins	Predispose to neuropathic pain identified
Capn7	128-3p	BMFC	Calpains are ubiquitous, have been implicated in neurodegenerative processes,	Critical for hippocampus-related contextual learning
	370-3p	KOC		Downregulated by Mild Stress in Rat Hippocampal Tissues
	128-3p	BMFC+KOC		Critical for hippocampus-related contextual learning
	370-3p			Downregulated by Mild Stress in Rat Hippocampal Tissues

	381-3p			Linked to insulin resistance or T2D
Sst	106b	BMFC+KOC	Neurotransmission	Protect mitochondrial functions
Satb2	128-3p	BMFC+KOC	Memory	Critical for hippocampus-related contextual learning
	148a-5p			Inhibition induces hepatocellular tumorigenesis
	381-3p			Linked to insulin resistance or T2D
Ip6k1	148a-5p	BMFC+KOC	Neurotransmission	Inhibition induces hepatocellular tumorigenesis
	770-3p			Down-regulated in the hippocampus of Sprague-Dawley rats with temporal lobe epilepsy
Tph1	379-5p	BMFC & KOC	biosynthesis of serotonin	Identified in primary sensory neurons that are associated with neuropathic pain

Arc: activity-regulated cytoskeleton-associated protein; Cbr3: carbonyl reductase 3; Tshz3: teashirt zinc finger homeobox 3; Pla2g5: phospholipase A2, group V; Htr2c: 5-hydroxytryptamine receptor 2C; Mrps23: mitochondrial ribosomal protein S23; Capn7: calpain 7; Sst: somatostatin; Satb2: SATB homeobox 2; Ip6k1: inositol hexakisphosphate kinase 1; Tph1: tryptophan hydroxylase 1; BMFC: buttermilk fat concentrate rich in phospho- and sphingolipids; KOC: krill oil concentrate rich in omega-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid) and phospholipids; BMFC+KOC: combination of BMFC and KOC.

Table S7. Sequence of miRNAs primers selected for validation.

miRNA	**sequence**
rno-let-7f-5p	TGAGGTAGTAGATTGTATAGTT
rno-miR-106b-3p	CCGCACTGTGGGTACTTGCTGC
rno-miR-128-3p	TCACAGTGAACCGGTCTCTTT
rno-miR-146a-5p	TGAGAACTGAATTCCATGGGTT
rno-miR-148a-3p	TCAGTGCACTACAGAACTTTG
rno-miR-148a-5p	AAAGTTCTGAGACACTCTGACTC
rno-miR-191a-5p	CAACGGAATCCCAAAAGCAGCTG
rno-miR-195-3p	CCAATATTGGCTGTGCTGCTCCA
rno-miR-29a-3p	TAGCACCATCTGAAATCGGTTA
rno-miR-30e-3p	CTTTCAGTCGGATGTTTACAGC
rno-miR-370-3p	GCCTGCTGGGGTGGAACCTGGT
rno-miR-381-3p	TATACAAGGGCAAGCTCT
rno-miR-770-3p	GTGGGCCTGACGTGGAG
rno-miR-99a-5p	AACCCGTAGATCCGATCTTGTG
rno-miR-379-5p	TGGTAGACTATGGAACGTAGG

Table S8. Sequence of genes primers selected for validation.

Gene	Name	Sequence
Arc	rno-Arc-FW	AAAGCAGCAGCAAGATGGTT
	rno-Arc-REV	GAGTCTTGCCTCCTGTCCTG
Cbr3	rno-Cbr3-FW	GCTGCCCATAATGAAACCACA
	rno-Cbr3-REV	GTCTGGCCAACCTTCTCTCT
Tshz3	rno-Tshz3-FW	GCAGCACAGCCATTATCACG
	rno-Tshz3-REV	GGCCAGACTGTTGCTCATCT
Pla2g5	rno-Pla2g5-FW	CTTGGGCTGCCAGCATAAAC
	rno-Pla2g5-REV	GCAGCCGTAGAAGCCATAGT
Htr2c	rno-Htr2c-FW	CGGACGGGGTACAAAACTGG
	rno-Htr2c-REV	AATCCAGACGGGGCACAAAT
Mrps23	rno-Mrps23-FW	CACATGGTGTGGTTCCTCGG
	rno-Mrps23-REV	CTTAGCCCAACCCGTGACAT
Capn7	rno-Capn7-FW	AGTTCTCCTCGCAGTGCCTC
	rno-Capn7-REV	TTGGGCAGCTTCCTTGTAGTA
Sst	rno-Sst-FW	ACCCCAGACTCCGTCAGTTT
	rno-Sst -REV	CCAGGGCATCGTTCTCTGTC
Satb2	rno-Satb2-FW	AAAACCTCGACACCGACAAC
	rno-Satb2-REV	CCAACGAAGCAGTTCACAGA
Ip6k1	rno-Ip6k1-FW	ACCAAGGCTGCATCATTTTGAC
	rno-Ip6k1-REV	AAACACACATTGCGTTGGGG

Arc: activity-regulated cytoskeleton-associated protein; Cbr3: carbonyl reductase 3; Tshz3: teashirt zinc finger homeobox 3; Pla2g5: phospholipase A2, group V; Htr2c: 5-hydroxytryptamine receptor 2C; Mrps23: mitochondrial ribosomal protein S23; Capn7: calpain 7; Sst: somatostatin; Satb2: SATB homeobox 2; Ip6k1: inositol hexakisphosphate kinase 1; Tph1: tryptophan hydroxylase 1; FW: Forward; REV: Reverse.

GENERAL DISCUSSION

The investigations reported in this thesis contribute to elucidating the molecular and nutrigenomic actions of several minor components of the diet.

In the first publication pertinent to this thesis, we performed the first nutrigenomic study, with soy isoflavones, i.e. genistein and daidzein, in adipose tissue. The results show that isoflavones, administered in nutritionally-relevant amounts, have diverse yet modest effects on adipose tissue (known to be prominently involved in cardiometabolism). The relevance of this study builds on the fact that isoflavones are the subject of active research and current recommendations on their consumption are under intense scrutiny. Some investigations contend that soy isoflavones might act as tumor-promoting or tumor-inhibiting agents, because of their phytoestrogenic activities. However, there is consensus that estrogens exert complicated and poorly understood effects on cardiovascular health. In our study, soy isoflavones increased total cholesterol, while lowering triacylglycerol concentrations. As soy consumption brings about hypolipidemic effects (possibly due to its proteins), the true significance of the observed cholesterol increase is equivocal and deserves further investigation. In summary, this work adds further evidence to the notion that soy isoflavones have assorted effects (both positive and negative) on CMD risk factors. Keeping into account the moderate average exposure to such molecules, their impact on cardiovascular health is likely negligible, and soy and its isoflavones can be consumed as part of a balanced diet.

The second study included a human trial concerning hydroxytyrosol (HT, the foremost and most actively studied component of extra virgin olive oil) consumption, which aimed at verifying the current hypothesis that (poly) phenols are processed by the body as xenobiotics. If this was true, (poly) phenols such as HT stimulate stress- related cell signalling pathways that result in increased expression of genes encoding

cytoprotective proteins. In particular, Nrf2 (NF-E2-related factor 2) is a transcription factor that binds to the Antioxidant Response Element (ARE) in cells and, thus, regulates enzymes involved in antioxidant functions or detoxification such as thioredoxin reductase-1 and glutathione peroxidases. According to the hormesis theory, (poly) phenols paradoxically act on the Keap1/Nrf2/ARE signaling axis to produce additive increases in electrophilic signaling that result in the induction of Phase II enzymes and increased nucleophilic substrates, such as glutathione, thioredoxin, and NADPH. In brief, (poly) phenols likely exert indirect rather than direct antioxidant actions. Even though the Nrf2 hypothesis is gaining traction, there are no solid human data to confirm it.

In this study, we tested the activities of HT on Phase II enzymes expression in a double-blind, randomized, crossover, placebo-controlled trial. We reported that HT, administered at two different doses (in a Latin-square design) was well tolerated (as shown by the unaltered levels of hepatic enzymes), but did not significantly modify the expression of Phase II enzymes in peripheral blood mononuclear cells (chosen because they are easy to obtain and reflect many whole body genetic expressions). Careful literature analysis shows that, while biochemical, i.e. *in vitro*, verification of Nrf2-mediated hormesis is strong, human evidence is scant or non-existent. Of note, the activation of Phase II enzymes might be organ-specific, e.g. chiefly taking place in the liver. However, whether sustained hepatic Phase II enzymes activation occurs in humans would require biopsies and, therefore, is very difficult to ascertain for obvious ethical and practical reasons. In short, the "hormesis hypothesis" that (poly) phenol activate Phase II enzymes requires solid human confirmation that might be provided by future trials.

We also performed a study regarding the bioavailability of HT, i.e. urinary excretion. We used 24-h urine samples from the volunteers who participated in the Phase II enzymes study to assess the degree of HT absorption, metabolization, and excretion. Indeed, one important - yet often overlooked issue – in the nutraceutical field is that of absorption and/or bioavailability of the active principle(s). A notable example is that of resveratrol, which is not bioavailable even when ingested in large quantities. Our results show that HT is absorbed and excreted when given as an olive mill wastewater extract preparation. In particular, ~8 to 10% (as mole %) of the administered HT was recovered in urine, and we confirmed that most of it undergoes sulphation at the 3´ position (HT-S-3´). It is noteworthy that there is ample evidence of the absorption and excretion of HT via extra virgin olive oil use. Yet, because HT is employed as nutraceutical either alone or as part of a formulation, data on its bioavailability could greatly help the development of appropriate investigations. For example, our data accentuate how HT-S-3´ should be quantified in studies of HT as nutraceutical, to monitor compliance.

As mentioned, a major goal of this thesis was the identification of cellular, genetic, and molecular targets of micronutrients, to elucidate their mechanisms of action. One relevant mechanism of molecular regulation involves microRNAs (miRNAs), which exert important regulatory actions on gene expression not only under physiological circumstances, but also in disease. Indeed, miRNAs often target multiple functionally related genes and extensively interfere with biological processes, rendering them good candidates for therapeutic and/or nutraceutical interventions. Two divergent approaches are currently being studied: (1) miRNAs inhibition *via* pharmaceutical formulations and (2) the use of miRNAs mimics in therapy. Within this context, the potential role of plant-derived phenolic molecules (*e.g.* polyphenols) in miRNAs

modulation is being very actively investigated, but no clear-cut result has been published. Therefore, phenolic modulation of miRNAs becomes an attractive strategy to target several biological processes.

The study presented in this thesis comprises animal, cellular, and human data obtained by testing HT effects on miRNAs expression. We provide the first *in vivo* evidence that HT intake modulates specific miRNAs in the small intestine of mice. Some of these miRNAs are also modulated in other tissues, strengthening the causality of our findings. Notably, some of these data were confirmed in a human setting, showing (also for the first time) that, in humans, HT modulates the expression of certain miRNAs. Although the precise molecular mechanism underlying the changes in miRNA expression induced by HT remains to be elucidated, our data suggest that the use of small, low-molecular-weight molecules capable of modulating miRNAs´ functions might be a viable alternative or an adjuvant to the current pharmacologic arsenal targeting endogenous miRNAs.

Giordano *et al.* (2014) were the first ones to investigate the nutrigenomic actions of HT. Their results showed that HT – in nutritionally relevant amounts - is able to positively modulate the glutathione-driven antioxidant enzymatic machinery in adipose tissue, speculatively via homeostatic feedback. To further investigate the precise molecular mechanisms that explain several of HT's potentially healthful actions, we performed a proteomic study. Of note, we used stable isotope labeling with amino acids in cell culture (SILAC), which is being acknowledged as a robust tool in proteomic quantification. In particular, as data on the effects of HT on tissue proteome *in vivo* are limited, we employed MS-based quantitative proteomics, namely super-SILAC, to investigate differences in protein levels in adipose tissue and the liver, i.e. the most metabolically active tissues, of mice fed long-term with HT. We also analyzed

peripheral blood mononuclear cells (PBMCs) obtained from healthy volunteers supplemented with HT for one week. We show that long-term hydroxytyrosol supplementation has the potential to modulate protein expression in adipose tissue and the liver. These proteins include fatty acid binding protein, glutamate dehydrogenase, ornithine carbamoyltransferase, epoxide hydrolase, nucleoside diphosphate kinase, pyruvate carboxylase mitochondrial, aconitate hydratase, 4-hydroxyphenylpyruvate dioxygenase, carboxylesterase, catalase, fructose-biphosphate aldolase, superoxide dismutase [Cu-Zn], vinculin, myosin-9, cofilin-1, vimentin, and hemoglobin subunit beta-1, among others. In other words, we confirmed, at the proteome level, the homeostatic effects of HT suggested by the nutrigenomic study of Giordano *et al.* (2014).

The cardiovascular activities of HT are being vigorously investigated worldwide and even led to an EFSA-endorsed health claim. Conversely, the potential neuroprotective actions of this molecule are largely unexplored. Recent epidemiological evidence demonstrated that diabetes is a risk factor for Alzheimer's disease (AD) onset and development. Indeed, meta-analyses of longitudinal epidemiologic studies show that diabetes increases AD risk by 50% - 100%, being insulin resistance (IR) the main binding link between diabetes and AD. Recent prospective studies showed that the Mediterranean diet is associated with lower incidence of AD. We hypothesized that HT could exert beneficial effects on IR associated to AD and investigated its mechanisms of action in an astrocytic model of AD., i.e. the astrocytic cell line C6 challenged with Aβ(25-35). We reported that HT prevented the pronounced activation of mTOR (which mediates IR), thereby restoring proper insulin signaling. Our results also suggest that the preventive role of HT on Aβ(25-35)-induced cytotoxicity in astrocytes is moderated

by an increased HT-induced activation of Akt, which is mediated by the insulin signaling pathway. In short, we demonstrate that HT protects Aβ(25-35)-treated astrocytes by improving insulin sensitivity and restoring proper insulin-signaling. These data provide some mechanistic insight on the observed inverse association between olive oil consumption and prevalence of cognitive impairment.

Finally, the last two publications included in this doctoral thesis report the effect of polar lipids dietary supplementation (KOC, BMFC and a combination of both) in aged rats, and how these compounds could improve or prevent age-associated cognitive decline. Krill oil is an attractive source of n-3 FAs (a large proportion are incorporated into PC), being responsible for the synthesis of the neurotransmitter acetylcholine and linked to memory. Beneficial effects of krill oil have been described on cognitive function in the elderly. BMFC, a by-product obtained from butter manufacturing with a high content of MFGM, contains high proportions of PS and SM, which play important roles in mitochondrial membrane integrity, activity of postsynaptic receptors, and activation of Protein Kinase C in memory formation.

We studied insulin signaling, mitochondrial activity, and synaptic signaling because impaired glucose metabolism and mitochondrial decay greatly increase with age. We hypothesized that these compounds, due to their characteristics, could have beneficial effects on glucose metabolism and mitochondrial dysfunction. Our results showed that, even though all animal groups had signs of peripheral insulin resistance, improvement was clearer in the group supplemented with BMFC+KOC. These data are in agreement with other studies where KO and MFGM have been shown to reduce insulin levels in type 2 diabetes obese individuals. Insulin signaling improved both in the hippocampus and cerebral cortex, two brain regions known to be essential in cognitive processes. We also report an increase in ATP levels in hippocampus, whereas

no changes were observed in the cerebral cortex. This is coherent with the observation that n-3 fatty acids incorporate into the various brain structures in a non-random, but selected manner. The apparent increased energy status of the hippocampus might be due to an increase in mitochondrial biogenesis and, therefore, mitochondrial signaling markers were analyzed. Significant differences were found in genes and proteins involved in the electron transport chain of mitochondria. Morever, increased levels of PGC-1α and BDNF also confirm this hypothesis. BDNF facilitates synaptogenesis by inducing the mTOR pathway, and the combined supplementation favored mTOR activation in the hippocampus.

To investigate the nutrigenomic actions of theses bioactive polar lipids, we analyzed the expression of genes and miRNAs related to synaptic activity and/or neurodegeneration in the hippocampus. Several hippocampal gene transcriptional changes influence a wide range of learning and memory processes and could be related to cognitive decline. Very few studies with MFGM supplemention focused on brain function and none on the brain of aged animals.

We found 38, 58, and 72 differentially expressed genes in the groups supplemented with KOC, BMFC and BMFC + KOC, respectively. Our study is the first to evaluate miRNA expression in response to BFMC supplementation. The combination of KOC and BMFC modulated the hippocampal expression of 119 and 23 miRNAs. Functional analysis of differently expressed miRNAs and their potential target genes, suggest they play a role in important neuroactive processes. The modulation of some miRNAs could not be solidly correlated with a decrease in the expression of their respective predicted target genes validated here and, therefore, we could not prove causation. Finally, we also reported a significant reduction in hippocampal ceramide levels with BMFC + KOC supplementation. Ceramide accumulation in the brain results

in a deregulation of energy balance and leads to different disorders such as obesity, disturbance of glucose homeostasis, neurodegenerative diseases and AD.

In summary, our results show that the bioactive polar lipids concentrates studied could represent a good pharma-nutritional tool to help prevent the noxious consequences of age-related cognitive impairment. Dietary supplementation with these concentrates could contribute to maintain proper neuroactive gene and protein expression, in part through the modulation of certain miRNAs, and to help sustain a balanced energetic status within the hippocampus, favoring neuronal health and delaying cognitive decline associated to age-related disrepair.

CONCLUSIONS

The structure of the conclusion section is organized in accordance with the chapter sequence presented throughout this Doctoral Thesis:

- **Chapter 1: Soy Isoflavones.**

I. The nutrigenomic study performed here with these compounds was the first focusing in adipose tissue. The administration of nutritionally relevant amounts of soy isoflavones by young mice resulted in an overexpression of genes involved in the MAPK pathway, and of genes that regulate different aspects of lipid metabolism (Fabp9, NPY and Sphk1), as well as a repression of genes associated with the chemokine signaling pathway.

II. The administration of nutritionally relevant amounts of soy isoflavones triggers contradictory effects on circulating biomarkers, contributing to increased cholesterol and leptin levels and to a decrease in triglyceride plasmatic levels.

- **Chapter 2: Hydroxytyrosol**

III. The daily supplementation of two different concentrations of hydroxytyrosol (5 and 25 mg), during one week, to healthy volunteers did not produce significant changes in the expression of Phase II enzymes by PBMCs, showing that, in these cells, the beneficial effects described for this molecule do not occur through this signaling pathway. The few significant effects observed may be due to the short supplementation period of this pilot study and trials with longer supplementation times would be necessary to confirm these results. On the other hand, HT supplementation did not cause adverse effects, as no alterations were

found in several inflammation, lipid profile, hepatic enzymes and oxidation markers of CVD.

IV. HT is bioavailable in the doses supplemented to human volunteers. ~ 8 to 10% of HT metabolites were recovered in urine and the majority undergoes sulfation at position 3 (HT-S-3), which could play a relevant role in the biological activities described. HT-S-3 could be used as a reliable biomarker to monitor compliance of olive oil intake.

V. In mice, at a dose that closely approximates human intake, HT significantly changed the expression of several genes and proteins associated to oxidation-reduction processes and to several lipid metabolism-associated pathways. In addition, HT modulates specific miRNAs in the small intestine and in other tissues (e.g. miR-483-3p). In humans, miR-193a-3p was modulated by HT.

VI. With regards to neurodegenerative diseases, HT increased the viability of astrocytes simulating Alzheimer's disease, leading to improved insulin sensitivity and restoring insulin-signaling.

❖ Chapter 3: Bioactive polar lipids

VII. Dietary supplementation of aged rats - for three months - with bioactive polar lipids improves peripheral and central insulin resistance.

VIII. BMFC and/or KOC increased hippocampal ATP concentrations and increased the expression of several genes involved in the electron transport chain, suggesting an amelioration of mitochondrial damage. The n-3 fatty acids ingested as phospholipids increase BDNF brain levels favoring a correct

energetic state within neurons and facilitating both mitochondrial and protein synthesis necessary for synaptic plasticity.

IX. Supplementation with these compounds induced epigenomic and nutrigenomic changes, in part by targeting both genetic and epigenetic mechanisms through miRNA modulation, which may influence cognitive development or brain function.

X. BMFC and KOC conjunct supplementation was necessary for a significant reduction in ceramide levels, which play an important role in neuronal composition changes through the different stages of development and whose enhanced levels are associated with age-related decay.

General conclusion

The micronutrients studied in this PhD thesis modulate important physiological pathways, which can partially explain the health benefits attributed to particular diets. As the effects of micronutrients are necessarily modest in amplitude, we need to exert caution when extrapolating these results to human therapy. However, the appropriate formulation of well-studied micronutrients and science-based dietary advice should be relevant parts of public health policies and further ad-hoc studies should be funded and pursued.

CONCLUSIONES

La estructura de la sección de conclusiones se organiza de acuerdo a la secuencia de capítulos presentada a lo largo de esta Tesis Doctoral:

- **Capítulo 1: Isoflavonas de soja.**

I. Este es el primer estudio nutrigenómico realizado con estos compuestos en el tejido adiposo. La administración de cantidades nutricionalmente relevantes de isoflavonas de soja en ratones jóvenes dio como resultado una sobreexpresión de genes implicados en la vía MAPK y de genes que regulan diferentes aspectos del metabolismo lipídico (Fabp9, NPY y Sphk1), así como una represión de genes asociados con la vía de señalización de la quimioquina.

II. La administración de cantidades nutricionalmente relevantes de isoflavonas de soja conlleva efectos contradictorios sobre los biomarcadores circulantes, contribuyendo a un aumento en los niveles de colesterol y leptina y a una disminución en los niveles plasmáticos de triglicéridos.

- **Capítulo 2: Hidroxitirosol**

III. La suplementación diaria de dos concentraciones diferentes de hidroxitirosol (5 y 25 mg), durante una semana, a voluntarios sanos no produjo cambios significativos en la expresión de enzimas de Fase II en PBMCs, lo que demuestra que, en estas células, los efectos beneficiosos descritos para esta molécula no ocurren a través de esta vía de señalización. Los pocos efectos significativos observados pueden deberse al corto período de suplementación de

este estudio piloto y serían necesarios ensayos con tiempos de suplementación más largos para confirmar estos resultados. Por otro lado, la suplementación con HT no causó efectos adversos, ya no se encontraron alteraciones relevantes en varios marcadores de CVD, inflamación, oxidación, perfil lipídico y enzimas hepáticas.

IV. El HT es biodisponible en las dosis suplementadas a voluntarios sanos. Entre un 8 y un 10% de los metabolitos de HT se recuperaron en la orina y la mayoría sufre sulfatación en la posición 3 (HT-S-3). Este metabolito podría desempeñar un papel relevante en las actividades biológicas descritas por el HT y, además, podría usarse como un biomarcador fiable para supervisar el cumplimiento de la ingesta de aceite de oliva.

V. En ratones, la suplementación con HT, a una dosis aproximada a la ingesta humana, modificó significativamente la expresión de varios genes y proteínas asociados a procesos de oxidación-reducción y a varias vías asociadas al metabolismo de lípidos. Además, el HT moduló miRNAs específicos en el intestino delgado y en otros tejidos (por ejemplo, miR-483-3p). En humanos, el consumo de HT resultó en una modulacion del miR-193a-3p.

VI. Con respecto a las enfermedades neurodegenerativas, el HT aumentó la viabilidad celular, mejoró la sensibilidad a la insulina y restauró la vía de señalización de la insulina, en un modelo de astrocitos simulando la enfermedad de Alzheimer.

- **Capitulo 3: Lipidos polares bioactivos.**

VII. La suplementación dietética con lípidos polares bioactivos a ratas viejas, durante tres meses, contribuyó a una mejora en la resistencia periférica y central a la insulina.

VIII. La suplementación con BMFC y/o KOC contribuyó a un aumento de la concentracion de ATP en el hipocampo y a un aumento en la expresión de varios genes implicados en la cadena de transporte de electrones, lo que sugiere una mejora del daño mitocondrial. Los ácidos grasos n-3 ingeridos como fosfolípidos potenciaron un aumento en los niveles de BDNF en el cerebro, contribuyendo a un estado energético adecuado dentro de las neuronas y facilitando la síntesis mitocondrial y de proteínas necesarias para la plasticidad sináptica.

IX. La suplementación con estos compuestos indujo cambios epigenómicos y nutrigenómicos, en parte, a través de la modulación de miRNAs, que pueden influir en el desarrollo cognitivo o en la función cerebral.

X. La suplementación conjunta de BMFC y KOC fue necesaria para una reducción significativa en los niveles de ceramidas. Las ceramidas juegan un papel importante en los cambios de composición neuronal a través de las diferentes etapas de desarrollo y sus niveles elevados están asociados con el deterioro relacionado con la edad.

Conclusión general

Los micronutrientes estudiados en esta tesis doctoral modulan vías fisiológicas importantes, que pueden explicar parcialmente los beneficios para la salud atribuidos a diferentes dietas. Como los efectos de los micronutrientes son necesariamente modestos en amplitud, debemos tener precaución al extrapolar estos resultados a la terapia

humana. Sin embargo, el estudio exhaustivo de los efectos de los micronutrientes para su inclusión en formulaciones apropiadas y el asesoramiento dietético basado en la evidencia cientifica deberían ser partes relevantes de las políticas de salud pública, por ejemplo a través de la financiacion de más estudios como los aquí expuestos.